DESIGN, SYNTHESIS, CHARACTERISATION AND EVALUATION OF *THYMYDYLATE SYNTHASE X (THYX)* INHIBITORS AGAINST *Mycobacterium tuberculosis*

A Dissertation submitted to
The Tamil Nadu Dr. M.G.R Medical University
Chennai

In partial fulfillment of the requirements
for the award of the degree of

MASTER OF PHARMACY
IN
PHARMACEUTICAL CHEMISTRY

Submitted by
26108335

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY
COLLEGE OF PHARMACY
MADRAS MEDICAL COLLEGE
CHENNAI-600 003
APRIL 2012

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CERTIFICATE

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Dr. A. JERAD SURESH, M.Pharm., Ph.D.,
Principal
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Madras Medical College,
Chennai-600 003

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Dr. A. JERAD SURESH, M.Pharm., Ph.D., MBA.,

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This is to certify that **MANIKANDAN.A**, Post graduate student, Department of Pharmaceutical Chemistry, College of Pharmacy, Madras Medical College, Chennai-03 had submitted her protocol (Part B Application) _____ for the dissertation programme to the Animal Ethical Committee, Madras Medical College, Chennai- 600 003.

TITLE: "DESIGN, SYNTHESIS, CHARACTERISATION AND EVALUATION OF THYMYDYLATE SYNTHASE X (THYX) INHIBITORS AGAINST *Mycobacterium tuberculosis*"

The Animal Ethical Committee experts screened her proposal _____ and have given clearance in the meeting held on at Dean's Chamber in Madras Medical College.

Signature,

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Dedicated to almighty, Parents, teachers, & friends

INTRODUCTION

1. INTRODUCTION

1.1. Tuberculosis [1,2,3,4]

Tuberculosis (TB) is an airborne, chronic, deadly bacterial infection caused by *Mycobacterium tuberculosis* (MTB), an aerobic bacilli belonging to the family *Mycobacteriaceae*, first identified in 1882 by Robert Koch, which affects the lungs and other organs as well.

Need For Focus on Tuberculosis Disease:

The sixteenth global report on tuberculosis by WHO (published in 2011) says

- ✓ In 2010, there were 8.8 million (range, 8.5–9.2 million) incident cases of TB, 1.1 million (range, 0.9–1.2 million) deaths from TB among HIV-negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB.

- ✓ In 2009 there were almost 10 million children who were orphans as a result of parental deaths caused by TB.

- ✓ TB kills four people every minute some-where in the world and accounts about two million deaths per year.

- ✓ There were 3.2 million (range, 3.0–3.5 million) incident cases of TB and 0.32 million (range, 0.20–44 million) deaths from TB among women in 2010.

- ✓ About 13% of TB cases occur among people living with HIV.

- ✓ Total of 225 million new cases and 79 million deaths are expected from tuberculosis for the period between 1998 and 2030.

- ✓ Approximately 50% of India's population is reported to be tuberculin test positive.

Need for the New Target

- ✓ There is also an alarming increase in cases of TB caused by Multi-Drug Resistant (MDR-TB) strains, Extreme-Drug resistant (XDR-TB) strains and Total Drug Resistance (TDR-TB) strains of *Mycobacterium tuberculosis (Mtb*), due to inadequate drug therapy as a result of incorrectly selected medications (or) suboptimal drug dosing.

- ✓ Further *Mycobacterium tuberculosis* (Mtb) has been exacerbated by human immuno-deficiency virus (HIV) and their calamitous synergism, since both are destructive together than individually.

- ✓ Approximately 75-80% of HIV infected patients are co-infected with *Mycobacterium tuberculosis* (Mtb) and as a result 50-70% of HIV positive patients develop active TB. Thus TB is predominately a disease of the poor and we must be able to scale up the new strategies for combating the problem of TB treatment.

- ✓ The HIV epidemic will increase tuberculosis cases by at least 200,000 each year in India. If HIV spreads more rapidly, tuberculosis may become uncontrollable for a generation.

- ✓ Despite enormous efforts taken, no new drug has been introduced in the market for the past 40 years and almost all the drugs used for TB and combination therapy have also failed in many cases due to tolerance.

- ✓ The re-emergence of tuberculosis (TB) as a global health problem over the past few

decades, accompanied by the rise of drug-resistant strains of *Mycobacterium tuberculosis*, emphasizes the need for discovery of new therapeutic drugs acting on the new targets against this disease.

All these factors prompted us to search for new targets and new molecules as anti TB agents.

1.2. Medicinal Chemistry [5,6,7]

Medicinal chemistry is a discipline at the intersection of Chemistry and Pharmacology which involves design, drug synthesis and evaluation of pharmaceutical agents. The drug discovery process involves design, synthesize, characterization and evaluation of new chemical entities suitable for therapeutic use. It also includes the study of existing drugs, their biological properties and their quantitative structure activity relationship (QSAR).

Drug Discovery

Drug discovery is the identification of novel active compounds, often called "hits", which are typically screened to have desired biological activity on a particular target. They might be single or combinations of compounds (compound library). Sources of hits can come from natural sources, such as plants, animals, or fungi and also from synthetic chemical libraries, such as those created through combinatorial chemistry or historic chemical compound collections that are tested *en-masse* against the biological target in question.

Optimization

Next step in drug discovery involves further chemical modifications of the chemical entities in order to improve the biological and physiochemical properties of a given compound library. Chemical modifications can improve the recognition of binding site (selectivity) and binding geometries (Pharmacophores) of the candidate compounds, their affinities and pharmacokinetics, or indeed their reactivity and stability during their metabolic degradation, which exhibit maximum potency, most selectivity, best pharmacokinetics and least toxicity. This step improves Pharmacokinetic and Pharmacodynamic aspects of the compound library which is taken for further processes.

QSAR study involves mainly physical, chemical and molecular docking tools (CoMFA® and CoMSIA®), of compounds that gives the data in first and second order equations. There are many theories related to this and the most relevant being Hansch's analysis that involves Hammett electronic parameters, steric parameters and logP (lipophilicity) parameters as the basis for *insilico* drug design.

1.3 Drug Design [8,9,10]

Drug design is the inventive process of finding new medications based on the knowledge of the biological target. The drug is most commonly an organic small molecule which activates or inhibits the function of a biological target such as a protein or enzyme, which in turn results in a therapeutic benefit to the patient. In the most basic sense, drug design involves design of small molecules that are complementary in shape and charge to the biomolecular target to which they interact and therefore will bind to it. Drug design frequently but not necessarily relies on computer modeling techniques to identify a lead or an active molecule. This type of modeling is often referred to as *Computer-Aided Drug Design* (CADD) or *in-silico* process.

Hit identification – Docking combined with scoring function can be used to quickly screen large databases of potential drugs *in silico* to identify molecules that are quickly to bind to protein target of interest.

Lead optimization – Docking can be used to predict orientation of a ligand binding to a protein (also referred as binding mode or pose) which is used to design more potent and selective analogs.

Ligand or Guest - The complementary parent molecule which binds to the receptor. Ligands are most often small molecules, but could also be another biopolymer.

Receptor or host - The "receiving" molecule, most commonly a protein or other biopolymer.

Docking – Computational simulation of a candidate ligand binding to a receptor.

Binding Mode - The orientation of the ligand relative to the receptor as well as the conformation of the ligand and receptor, when bound to each other.

Pose- A candidate scoring mode.

Scoring- The process of evaluation of a particular pose by counting the number of favorable intermolecular interactions such as hydrogen bonds and hydrophobic contacts.

Ranking – The process of classifying which ligands are most likely to interact favorably to a particular receptor based on the predicted free energy of binding.

1.3.1 Types

There are two major types of drug design.

1. Ligand-based drug design
2. Structure-Based Drug Design (SBDD).

Ligand based drug design (Pharmacophore modeling)

Ligand-based drug design (or indirect drug design) relies on knowledge of other molecules that bind to the biological target of interest. These other molecules may be used to derive a pharmacophore model which defines the minimum necessary structural characteristics a molecule must possess in order to bind to the target. In other words, a model of the biological target may be built based on the knowledge of what binds to it and this model in turn may be used to design new molecular entities that interact with the target. Alternatively, a Quantitative Structure-Activity Relationship (QSAR) in which a correlation between calculated properties of molecules and their experimentally determined biological activity may be derived. These QSAR relationships in turn may be used to predict the activity of new analogs.

Pharmacophore model can be developed in two ways by using HIPHOP and HYPOGEN. HIPHOP provides feature based alignment without considering activity where as HYPOGEN generates activity based pharmacophore model which can predict the activity of new compounds. To run a HYPOGEN we need large number (more than 15) of molecule with known activity but we have very few molecules to inhibit *Thymidylate syntheses (ThyX)* of *Mycobacterium tuberculosis*. So we resorted to HIPHOP modeling to suggest the possible alignment of active molecules which can generate a pharmacophore model in early stages of project from only a small set of compounds of known or unknown activities.

Structure based Drug Design (Molecular docking)

Structure-based drug design (or direct drug design) relies on knowledge of the three dimensional structure of the biological target obtained through X-ray crystallography or NMR spectroscopy. Structural features of the target allow us to design a drug candidate with suitable physiochemical properties necessary for the interaction with the biomolecule. If an experimental structure of a target is not available, then also it is possible to create a homology model of the target protein based on the experimental structure of an available related protein. Using the structure of the biological target, candidate drugs that are predicted to bind with high affinity and selectivity to the target may be designed using interactive graphics and the intuition of a medicinal chemist. Alternatively various automated computational procedures also used to suggest new drug candidates.

1.3.2 Docking

Docking is frequently used to predict the binding orientation of drug candidates to their protein targets in order to predict the affinity and activity of the small molecule. Glide is a ligand docking program that predicts the protein-ligand binding modes and ranking the ligands via high-throughput virtual screening. Glide utilizes two different scoring functions called Standard Precision (SP) mode and Extra Precision (XP) mode Glide scores, to rank order the compounds based on the interaction with the receptor. Different sampling ligand conformational and positional degrees of freedom are available to determine the optimal ligand orientation relative to rigid protein receptor geometry.

The quality of any docking results depends on reasonable starting structures for both the protein and the ligand. The protein and ligand structures need to be prepared to achieve the best docking results. Following steps are involved in docking process.

Protein and preparation

A typical PDB (Protein Data Bank) structure file can be multimeric consists of heavy atoms and can contain waters, cofactors and metal ions. The structure generally has no information on bond orders, topologies, or formal atomic charges. Terminal amide groups can also be misaligned, because the X-ray structure analysis cannot usually distinguish between O and NH_2. Ionization and tautomeric states are also generally unassigned.

The following processes are necessary to make protein to perfect structure for docking study:

1. Assign ionization and tautomeric states of protein properly. (Side chains are reoriented when necessary and steric clashes are relieved).
2. Delete all water molecules (except those coordinated to metals, if water molecules are kept, hydrogen's will be added to them).
3. Adjust the protonation of the protein, which is crucial when the receptor site is a metalloprotein.
4. Finally minimize the protein to reorient side-chain hydroxyl groups and alleviate potential steric clashes present in the PDB structure.

Ligand Preparation

To give the best results, the structures that are docked must be good representations of the actual ligand structures as they would appear in a protein-ligand complex. Most of the docking tools modify the torsional internal coordinates of the ligand during docking, so the rest of the geometric parameters must be optimized beforehand.

This means that the structures supplied to docking tool must meet the following conditions:

1. They must be three-dimensional (3D).

2. They must have realistic bond lengths and bond angles.

3. They must each consist of a single molecule that has no covalent bonds to the receptor, with no accompanying fragments, such as counter ions and solvent molecules.
4. They must have all their hydrogen's valences filled.
5. They must have an appropriate protonation state for physiological pH values.

Receptor grid generation

Position and size of binding site in the receptor was represented in this step. Ligand docking cannot be performed until the receptor grids have been generated. This process was done by the tool (Receptor Gride generation) of maestro 9.1, after the protein preparation process.

Ligand Docking

The docking process calculates the binding energies of a particular conformational sample ligand with the receptor. Accessing the affinity of various ligands with the particular receptor was carried out by Glide by extra precision mode (XP).

1.3.3 Binding Site Analysis

Understanding the structure and function of protein binding sites is the cornerstone of structure-based drug design and this requires knowledge of both the location and physical properties of the binding site. In addition, the identification of small-molecule binding sites as modulators of protein-protein interactions is of increasing interest. Furthermore, even when a validated binding site has been identified, it is often important to find additional potential binding sites where appropriate targeting could result in different biological effects or new classes of compounds. When the binding site is not known from a 3-D structure or from other experimental data, computational methods can be employed to suggest likely locations. Medicinal chemistry efforts to give better ligands in case of receptor with well known binding site. Critical assessment of degree to which the occupancy of accessible but unexplored regions of receptor binding site for the interaction with ligand can be promoted by improving the

physical properties of the ligand. Such assessments can assist in the evaluation and optimization both of known binding molecules and of virtual screening hits. It is also important to understand the potential drugs ability of the site.

1.3.4 Scoring Methods [11]

Scoring of docked poses is still regarded as one of the major challenges in the field of molecular docking. The purpose of the scoring procedure is the identification of the correct binding pose by its lowest energy value and the ranking of protein-ligand complexes according to their binding affinities. Scoring functions can be divided in empirical scoring functions, scoring functions derived from force fields and knowledge-based scoring functions. Scoring functions derived from force fields handle the ligand binding prediction with the use of potential energies (non-bonded interaction terms) and sometimes in combination with solvations and entropy contributions. Knowledge-based scoring functions are based on atom pair potentials derived from structural databases. Forces and potentials are collected from known protein-ligand complexes to get a score for their binding affinities.

1.4 Scope

Today, CADD and screening methods have become the basis of new drug discovery. As the computational technologies advance there is increased efficiency of the drug discovery processes, minimized time duration and resource needs. In addition, as the knowledge of structural information of potential therapeutic targets dramatically expands, it drives the development of the new computational methodologies with greater automation, faster algorithms and improved information management techniques. On a genomic scale, instead of looking at individual targets, families of related targets will be studied. The information available on ligand binding to these families will be vastly expanded and used for discovery process.

The job of the molecular modeler is to handle the data effectively, as well as translate the available structural information into a form directly usable by the bench chemist. This mission

will ultimately cause a greater interface of bio and chemoinformatics, leading to improved structural and functional genomics knowledge.

1.5 Absorption, Distribution, Metabolism, Excretion (ADME) Analysis

Usually drug discovery and development are expensive and time-consuming processes. In pharmaceutical research many new drug failures which occur in the phase of clinical trials are due to ADME properties. This has to be identified early in the drug discovery process. Thus, *in-vitro* approaches are now widely used to investigate the ADME properties of new chemical entities and more recently, computational (*in silico*) modeling has been investigated as a tool to optimize selection of the most suitable drug candidates for development.

The objective of *in-silico* modeling tools for predicting these pharmacokinetic properties is to serve two key aims. First, at the design stage of new compounds and compound libraries so as to reduce the risk of late-stage attrition; and second, to optimize the screening and testing by looking at only the most promising active compounds.

***Lipinski's rule-of-five*:** The properties which can differentiate drugs from other chemicals can be considered as drug like properties. The crucial properties of the chemical candidate for oral delivery (Lipinski's rule-of-five) includes molecular mass <500 Daltons (Da), calculated octanol / water partition coefficient (CLOGP) <5, number of hydrogen-bond donors <5 and number of hydrogen-bond acceptors <10. These properties are then typically used to predict ADME models and form the basis for what has been called property-based drug design.

A deeper understanding of the relationships between ADME parameters with molecular structure properties has been used to develop *in silico* models that allow the early estimation of several ADME properties of the newer candidates. Among other important issues, prediction of properties that provide information about dose size and dose frequency such as oral absorption, bioavailability, brain penetration, clearance (for exposure) and volume of distribution are (for frequency) also needed for such drugs.

Prediction of ADME and related properties

Absorption: For a compound crossing a biological membrane by purely passive diffusion, a reasonable permeability estimate can be made using single molecular property, such as log D (Diffusion coefficient) or hydrogen-bonding capacity. The simplest *insilico* model for estimating absorption are based on a single descriptor, such as log P (Partition coefficient) or log D, or polar surface area, which is a descriptor of hydrogen-bonding potential. Different multivariate approaches, such as multiple linear regressions, partial least squares and artificial neural networks, have been used to predict quantitative structure–human-intestinal-absorption relationships.

Bioavailability: Important properties for determining permeability (Bioavailability) seem to be the size of the molecule, its capacity to make hydrogen bonds, its overall lipophilicity, its shape and flexibility.

Blood–brain barrier penetration*: (*BBB) Drugs to act in the CNS need to cross the blood–brain barrier (BBB) to reach their molecular target in the brain. By contrast, for drugs with a peripheral target, BBB penetration might not be required in order to avoid CNS side effects. Lipinski rule-of-five recommendations regarding the molecular parameters that contribute to the ability of molecules to cross the BBB have been made to aid BBB-penetration predictions.

Example: molecules with a molecular mass of <450 Da or with Polar surface area (PSA) <100 Å are more likely to penetrate the BBB along with other drug like parameters.

Dermal and ocular penetration: The existing octanol/water partition coefficient models are typically a function to estimate aqueous solubility, including hydrogen-bonding parameters, molecular weight and molecular flexibility. *Insilico* commercial models for the prediction solute-permeation rates through the skin are available, like the QikProp and DermWin programs.

Metabolism: *In silico* approaches also available to predict metabolism can be divided into QSAR and three-dimensional- QSAR studies, protein and pharmacophore models and predictive databases. Predictive-metabolism tools currently used require considerable input from a computational chemist and some other tools also be used as rapid filters for the screening of virtual libraries. Perhaps the most intellectually used and satisfying molecular modeling studies are those based on the crystal structure of the metabolizing enzymes. Several approaches that use databases to predict metabolism are available. Ultimately, such programs might be linked to computer-aided toxicity prediction on the basis of quantitative structure–toxicity relationships and expert systems for toxicity evaluation.

1.6 *In silico prediction* of toxicity issues

Toxicity is responsible for ~20–40% of drug failures to reach the market and for the withdrawal of a significant number of compounds from the market once they have been approved. Commercially *in-silico* tools are available and can be used for forecasting potential toxicity issues, they were roughly classified into two groups. The first approach uses expert systems that derive models on the basis of abstracting and codifying knowledge from human and the scientific literature sources. The second approach relies on the generation of descriptors of chemical structure and statistical analysis of the relationships between these descriptors and the toxicological end-point.

Current software packages are primarily emphasis on carcinogenicity and mutagenicity, although some packages do also include tools and/or knowledge bases for other end-points, such as teratogenicity, irritation, sensitization, immunotoxicology and neurotoxicity. There is an unmet need for *in-silico* predictive toxicology software for other end-points important in drug development, such as QT prolongation hepatotoxicity and phospholipidosis.

1.7 ANTITUBERCULAR ACTIVITY

In-vitro methods are laboratory experiments which are used as a confirmatory tool for the synthesized compounds. Every synthesized chemical entity should be evaluated *in-vitro* and *in-vivo* for the conformation of the expected activity.

***In-Vitro* assays**

- ✓ MB 7H10 Agar dilution Assay
- ✓ Micro plate Alamar Blue Assay (MABA)
- ✓ Resazurin Microtitre Assay (REMA)
- ✓ Luciferase Reporter Phage Assay (LRP)
- ✓ Serine/Threonine Protein (STP) Kinase Assay
- ✓ BACTEC-TB system

***In-vivo* assays**

- ✓ Balb/C mouse model for CFU and mortality.

1.8 Toxicological Evaluation

Principle and Purposes

Acute toxicity tests the toxicity of a chemical or a drug substance after a single oral administration.

The main purpose of this study is to evaluate the degrees of toxicity in a quantitative and qualitative manner to compare it with the existing drug candidates. Further acute toxicity study provides the information about the acute toxicity effects of a chemical in a quantitative manner i.e. it gives information about the mechanism of acute toxicity.

The method of evaluation has been changed in the last three decades mainly for animal welfare reasons. Producing mortality in animals in order to determine LD_{50} (dosis letalis media) is no longer the main purpose of acute toxicity study. Nowadays acute toxicity study mainly focuses on acute tolerance, nature of acute toxicity, symptoms in sub-lethal range and dose levels which acute mortality in few animals. i.e quality has replaced quantity.

The test is based on stepwise procedure with use of minimum number of animals per step, sufficient information is obtained on the acute toxicity of the test substance to enable its classification.

The drug substance is administered orally to a group of experimental animals at one of the defined dose. The drug is tested using a stepwise procedure, each step using three animals of a single sex (normally females). Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step i.e.

- No further testing is needed
- Dosing of three additional animals, with same dose,
- Dosing of three additional animals at the next higher or the next lower dose levels.

LITRATURE REVIEW

Literature Review

The literature was reviewed to gain insight about the disease, drug design of ligands, synthesis, characterization, and methods of *invitro* evaluation.

Drug design

1. **Damien Leduc et al.,** [11] (2007) *Thymidylate Synthase (ThyX*) role in de nova folate synthetic pathway in microorganism were established. Flavin-dependent *ThyX* proteins show *thymidylate synthase* activity in vitro and functionally complement *ThyA* defects in heterologous systems, direct proof of their cellular functions is missing.

2. **Eric M. Koehn et al.,** [12] (2009) Thymidylate biosynthesis pathway in ThyX gene containing micro organism were characterized. Biosynthesis of the DNA base thymine depends on activity of the enzyme *Thymidylate Synthase (TS)* to catalyze the methylation of the uracil moiety of 2'-deoxyuridine-5'-monophosphate (dUMP).
   known *Thymidylate Synthases (TSs*) rely on an active site residue of the enzyme to activate dUMP1.

3. **Damien Leduc et al.,** [13] (2004) Acting binding site in the *Thymidylate Synthase (ThyX)* was established. Identified several residues of the *Helicobacter pylori* ThyX protein with crucial roles in *ThyX* catalysis. By providing functional evidence that the active site(s) of homotetrameric *ThyX* proteins is formed by three different subunits, our findings suggest that ThyX proteins have evolved through multimerization of inactive monomers. Moreover, because the active-site configuration of *ThyX* proteins, present in many human pathogenic bacteria and of human *thymidylate Synthase.*

4. **F. Esra Önen et al.,** [14] (2008) potent inhibitors (Thiazolidine derivatives) against *Thymidylate Synthase (ThyX) was* designed, synthesized. Three synthesized series of compounds based on a thiazolidine core allowed identification of potent inhibitors of *thymidylate synthase X*. The evaluation of the catalytic activity of the enzyme in the presence of these molecules revealed two distinct classes of compounds that inhibit

ThyX with submicromolar concentrations, which could lead, after optimization, to effective inhibitors with potential biomedical interest.

5. **Joshua H. Hunter et al., [15]** (2008) Enzyme Kinetics and ligand binding preferences of *Thymidylate Synthase* both *ThyX* and *ThyA* in *Mycobacterium tuberculosis* were characterized. Cloning, over expression, and purification, the thymidylate-synthesizing ability of *ThyA* and *ThyX* gene products were directly confirmed by HPLC analysis of reaction products and substrate saturation kinetics were established. 5-Fluoro-29-deoxyuridine 59-monophosphate (FdUMP) was a potent inhibitor of both *ThyA* and *ThyX,* offering important clues to double-targeting strategies

6. **Martin Kogler et al., [16] (**2011) synthesized and Evaluated 5-Substituted 2'-deoxyuridine Monophosphate Analogues As Inhibitors of Flavin-Dependent Thymidylate Synthase in Mycobacterium tuberculosis. Series of 5-substituted 20-deoxyuridine Monophosphate analogues has been synthesized and evaluated as potential inhibitors of mycobacterial *ThyX,* a novel flavindependent *thymidylate synthase* in Mycobacterium tuberculosis. Their SAR estabilished.

7. **M. Paola Costi et al., [17] (**2006) developed anti microbial drugs new 1,2-naphthalein derivatives have been synthesized and tested against a TS-based biolibrary, including *human Thymidylate synthase* (hTS)

8. **Ahmed Haouz et al., [18]** (2003) synthesized and structurally analyzed inhibitors against *Thymidylate kinase* in *mycobacterium tuberculosis.* The chemical synthesis of new compounds designed as inhibitors of *Mycobacterium tuberculosis TMP kinase* (*TMPK*) is reported. The synthesis concerns TMP analogues modified at the 5-position of the thymine ring as well as a novel compound with a six-membered sugar ring.

9. **Jonathan E. Ulmer et al., [19]** (2008) functionally analyzed and revealed an extended motif of amino acids essential to enzyme activity in *M. tuberculosis*

10. **Fre´de´ ric Escartin et al.,** [20] (2008) studied chromosomal DNA replication in *Thymidylate Synthase* containing organisms. Demonstrate that DNA replication speed in bacteria and archaea that contain the low-activity *ThyX* enzyme is up to 10-fold decreased compared with species that contain the catalytically more efficient *ThyA*.

11. **Hui-Yuan Wang et al.,** [21] (2008) Pharmacophore modeling and virtual screening for designing potential PLK1 inhibitors. The best quantitative Pharmacophoremodel, Hypo1, which has the highest correlation coefficient (0.9895), consists of one hydrogen bond acceptor, one hydrogen bond donor, one hydrophobic feature, and one hydrophobic aliphatic feature.

12. **Sugunadevi Sakkiah et al.,** [22] (2011) Pharmacophore based Virtual Screening, molecular docking studies to design potent heat shock protein 90 inhibitors. The best hypothesis from Hip-Hop,Hypo1, one hydrogen bond donor (HBD), two hydrogen bond acceptors (HBA), and two hydrophobic (H) and structure-based hypothesis, SB_Hypo1, one HBA, one HBD and four H features, were generated using Discovery Studio and Ligand Scout, respectively.

13. **Mohammad Neaz Morshed et al.,** [23] (2010) Computational approach to the identification of novel Aurora-A inhibitors. A three-dimensional common feature Pharmacophore model was developed with the HipHop program provided in the Catalyst® software package and this model was used as a query for screening the databases

Synthesis

14. **Mohit L. Deb and Pulak J. Bhuyan** [24] (2005) Uncatalysed Knoevenagel condensation in aqueous medium at room temperature. Knoevenagel condensation of various aromatic and heteroaromatic aldehydes with active methylene compounds like malononitrile, ethyl cyanoacetamide, ethyl cyanoacetate, barbituric acids, Meldrum_s

acid, dimedone and pyrazolone proceeds smoothly with stirring in an aqueous medium.

15. **Khalafi-Nezhad, Ali* and. Hashemi, Aboulghasem** [25](2001) Microwave enhanced Knoevenagel Condensation of barbituric acid with aromatic aldehydes on basic aIumina. Efficient Knoevenagel condensation of barbituric acid with different aromatic aldehydes on basic alumina was performed in a conventional microwave ovenin the absence of solvent.

16. **Ina Bolz et al.,** [26] Novel Schiff bases derived from 5-aminobarbituric acid: synthesis and solid state structure. Synthesis and characterization of novel Schiff bases with multiple binding sites for supramolecular assemblies are presented. For this purpose 1,3-dimethyl- and 1-butyl-5-aminobarbituric acid are condensed with *para*-nitro- and *para-N,N*-dimethylaminocinnamaldehyde, respectively

17. **Palwinder singh et al.,** [27] (2005) Microwave synthesis of 5-acyl barbituric acid derivatives. Synthesis and characterization of novel Schiff bases with multiple binding sites for supramolecular assemblies are presented. For this purpose 1,3-dimethyl- and 1-butyl-5-aminobarbituric acid are condensed with *para*-nitro- and *para-N,N*-dimethylaminocinnamaldehyde, respectively

18. **Kelsey C. Miles et al.,** [28] The Clauson-Kaas pyrrole synthesis under microwave irradiation 10-30 min using water, acetic acid as medium.

19. **Da David J. Guerin et al.,** [29] (1999) Chemical modification of Benzyloxybenzyl-barbituric acid and its effects on urdpase – synthesized uridine phosphorylase inhibitors.

20. **James J.-W. Duan et al.,**[30] (2005) synthesized Non-hydroxamate 5-phenylpyrimidine-2,4,6-trione derivatives as selective inhibitors of tumor necrosis factor-*a* converting enzyme.

21. **Jie Jack Li et al.,**[31] (2007) synthesized Quinazolinones and Pyrido[3,4-*d*]pyrimidin-4-ones as orally active and Specific Matrix Metalloproteinase-13 inhibitors for the treatment of Osteoarthritis.

Invitro activity

22. **Clarice Queico at al.,** [32] (2000) Standartization of Broth Microdilution Method for *Mycobacterium tuberculosis*

23. **Ahmet Yilmaz Coban et al.,** [33] (2004) Drug susceptibility testing of *Mycobacterium tuberculosis* by the Broth Microdilution method with 7H9

24. **Claude Kirimuhuzya et al.,** [34] The anti-mycobacterial activity of *Lantana camara* a plant traditionally used to treat symptoms of tuberculosis.

AIM

&

OBJECTIVE

Aim and Objective

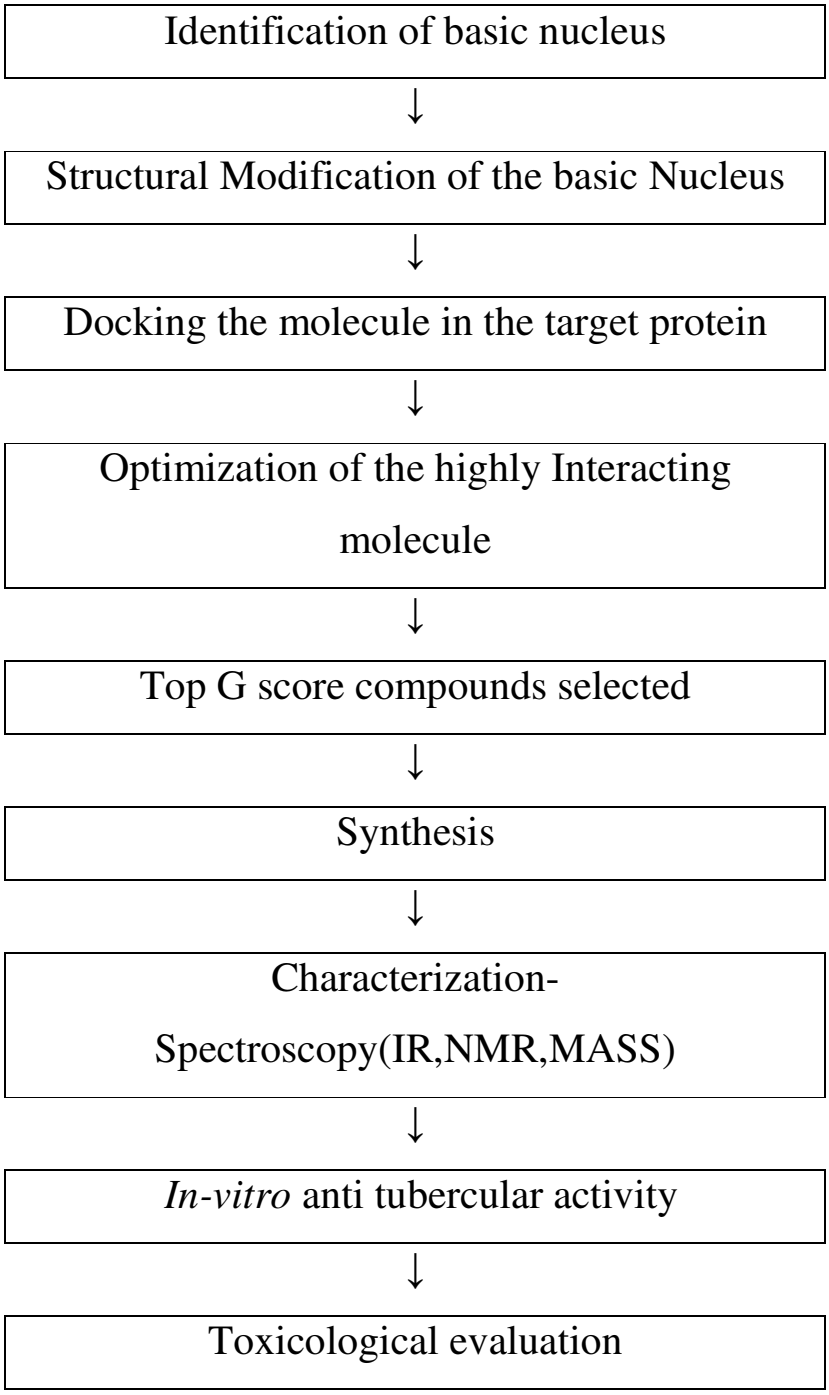
Aim

To design and synthesize effective Antitubercular agents against *Thymidylate Synthase X (ThyX)* of *Mycobacterium tuberculosis*

The objectives of study include

1. *In-silico* design of *Thymidylate Synthase (ThyX)* inhibitors.
2. Laboratory synthesis of the designed compounds.
   - ✓ 5-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl] furan-2-sulfonic acid.
   - ✓ 5-{(*Z*)-[(2, 4, 6-trioxohexahydropyrimidin-5-yl) imino] methyl} furan-2-sulfonic acid.
   - ✓ 5-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl] furan-2-sulfonic acid.
   - ✓ 2-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl]-4*H*-imidazole-4-sulfonic acid.
3. Characterization of the above compounds by Infrared Spectroscopy, Nuclear Magnetic Resonance and Mass Spectroscopy.
4. *In-vitro* Antitubercular activity of synthesized compounds.
5. Toxicological evaluation.

The present study was conducted in according to the following design.



MATERIALS

&

METHODS

4. MATERIALS AND METHODS

The entire processes comprises of

- Drug Design
- Synthesis
- Characterization
- *In-vitro* Antitubercular activity
- Acute toxicity study

4.1 DRUG DESIGN

A binding interaction between a drug molecule and a target enzyme results in inactivation or inhibition of the enzyme. If the protein is a receptor, ligand binding may result in agonism or antagonism. Docking is suitable method in the field of drug design to predict.

4.1.1 Glide Docking [35]

Using different heterocyclic nucleus large data bases of compounds were prepared and docked against *Thymidylate Synthase* of *Mycobacterium tuberculosis* using Glide Software (Maestro 9.1). Extra precision (XP) scoring function was utilized to rank order the compounds. This process was carried out in three steps.

Identification of basic nucleus

Very few compounds have been reported against this Thymidylate Synthase X (ThyX) target. Structurally similar molecules with predicted ThyX activity were chosen for synthesis and then docked. From the docking result nucleus which has more interaction with the binding site are taken for further modifications.

Design of new ligand

Selected basic nucleuses were further substituted with various functional groups and more than 2500 newer ligands are synthesized and docked against the *Thymidylate Synthase X* (*ThyX).* From this high scoring molecules are optimized in the next step.

Optimization of the ligand

Final optimization of the ligand in needed to eliminate the toxic functional groups and minor modifications carried out to get the better ligand-target interaction to inhibit the protein. Highest scoring 20 ligands are taken regardless of their basic nucleus and their structures are modified by inter changing the position of the functional groups, docked in the receptor. More than 200 compounds are designed in this step and docked against Thymidylate Synthase (ThyX). For optimization of the ligands HIPHOP qualitative study and 2D Similarity searching were used

Docking score method

The compounds ranked based on G score which the basic value is obtained by the consideration of both rewards and penalties when the molecule interacted with the target.

Rewards

Lipophilic pair term and fraction of the total protein ligand Vanderwaals energy, hydrophobic enclosure reward, hydrophobically packed hydrogen bond, hydrophobically packed correlated hydrogen bond, hydrogen bond pair term, electrostatic rewards, sitemap ligand/ receptor, Pi and cation, chlorine and bromine, low molecular weight these all are basic parameters consider as rewards of the docking score. Rewards values are noted in negative values.

Penalty

Ligand with large hydrophobic contacts and low hydrogen bond scores, exposed hydrophobic ligand groups, rotatable bond, similarity these are the basic penalty parameters of the docking score. The penalty values should be less in values to indicate best compounds.

4.1.2 HIPHOP generation [36-40]

Selecting proper orientation of the molecule will have an impact on pharmacophore model. Hip-hop is a qualitative method which is used to align a set of molecules based on their common chemical features. A minimum of 2 to a maximum of 32 compounds with or without activity data is taken as the training set because HIPHOP will consider only common chemical

features. Here we taken 6 molecules of known activity to determine 3D spatial arrangements that are common to molecules in the training set. HIPHOP generates feature based 3D-Pharmacophoric alignment in 3 steps.

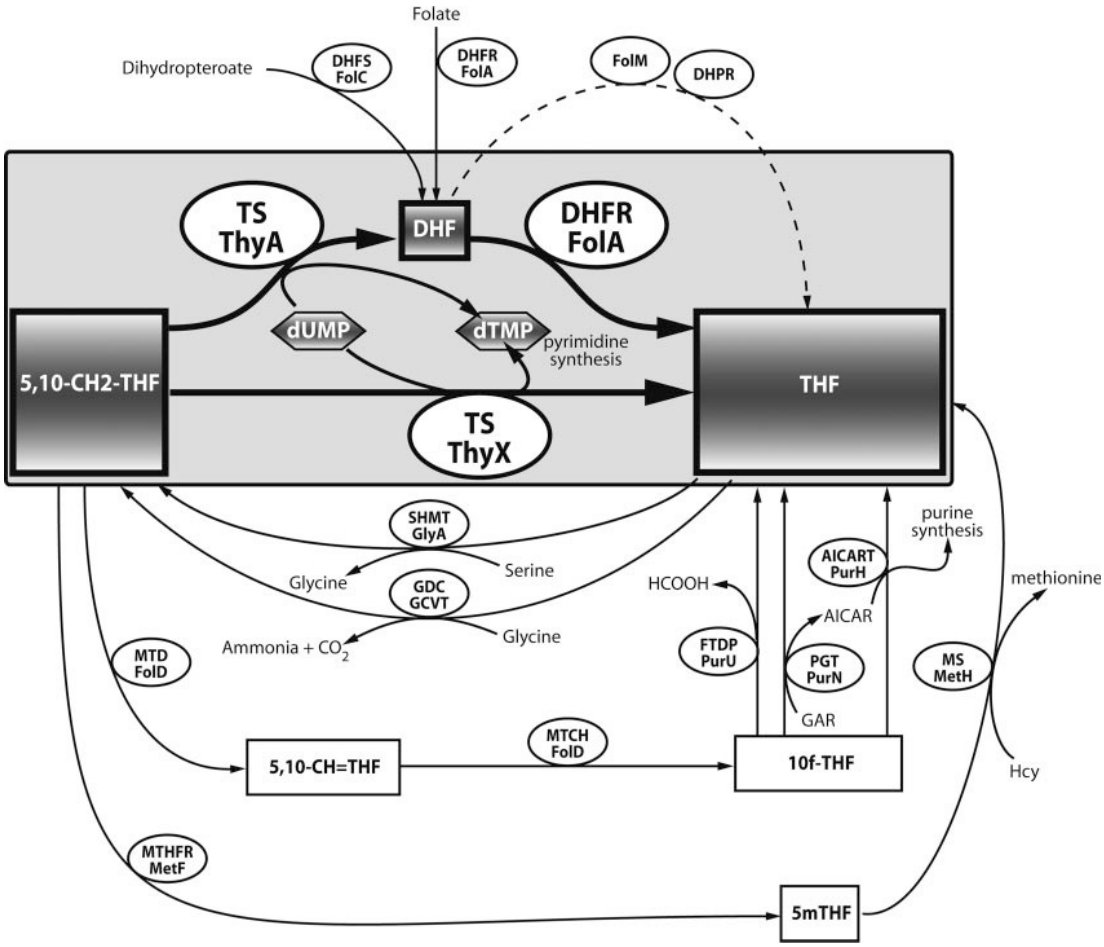
1. A conformation model for each molecule in the training set is generated
2. Each conformer is examined for the presence of chemical features.
3. A 3D configuration of chemical features common to input molecules were determined.

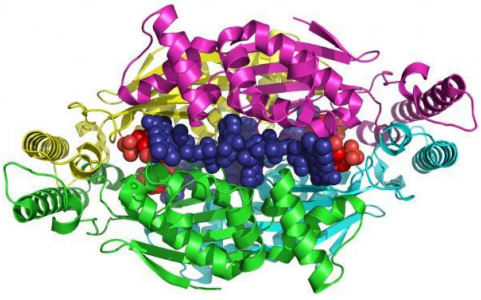
Catalyst containing a large number of features like, H-bond donor, H-bond acceptor, Hydrophobic (both aliphatic and aromatic), Ionizable groups (both positive and negative) which can be selected for the HIPHOP hypothesis generation. The features which are present in the training set molecules are only selected. For this feature selection knowledge of ligand-receptor interaction should be included and the minimum numbers of features are included. After the completion of the hypothesis, H-bond donor, H-bond acceptor and negative ionizable groups are found as common features in the training set with their inter-feature distances were obtained. Based on these features new molecules were screened to find the common features present in it. From the large quantity of the designed molecules, 33 were found with same common features as in the training set and they were taken for the next step.

4.1.3 2D similarity searching

A molecular similarity searching technique based on atom environments, information-gain-based feature selections present in the molecule. 2D fragment based similarity searching is one of the popular technique to find a lead from a large data base of chemical structures and 2D-fragment based similarity searching found best in terms of hit rate than other methods. The molecules having known activity were taken as reference compound and the designed ligands which have similar physiochemical features of that reference molecule are screened as percentage calculation. We chosen 5-Substituted 2'-deoxyuridine Monophosphate Analogues as the reference molecules and the designed molecule contains more similarity were screened.

4.2 Mechanism: Antitubercular activity [41, 42]





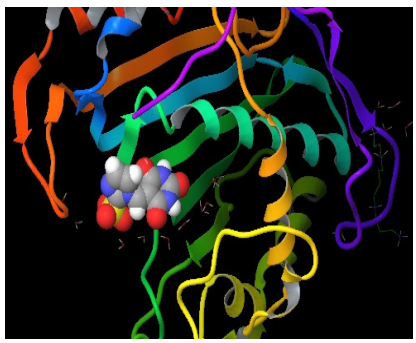


Figure 4.1 ThyX Protein and its mechanism

Mechanism of Action of *Thymidylate Synthase X (ThyX)* Activity

- The Folate cycle plays a central role in metabolism. Folate dependent enzymes are required for methylation reactions and synthesis of purine and pyrimidine nucleotides and many amino acids.

- Many folate cycles are interconnected and the basic model has the qualitative behavior seen in folate homeostasis in the cytosol of human cells.

- In DNA synthesis, dividing cells require large quantities of DNA precursor *Thymidylate (dTMP).* In human cells *Thymidylate Synthase (ThyA)* catalyses the reductive methylation of (dUMP) to (dTMP). Using 5, 10-methylene tetrahydrofolate (CH_2THF) as a donor of one carbon units and as a reductant.

- Formation of dTMP is rate limiting for DNA replication in human cells.

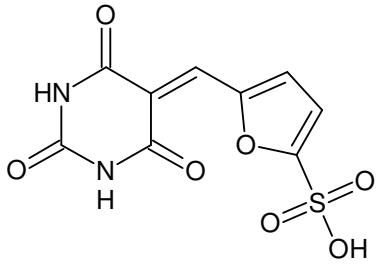
- Differently from human *Thymidylate Synthase ThyA*, the members of the novel family of Thymidylate Synthase ThyX are NADPH oxidases that use flavin adenine nucleoside (FAD) to mediate hydride transfer

- Both thyA, thyX catalyses formation of Thymidylate in *invitro* their reductive mechanism is different. thyX catalyses results in formation of THF and not DHF as the product of the methylation reaction but virtually nothing is known to rate about how the activity of flavin dependent Thymidylate Synthase (ThyX) influences the different folate-dependent branches of the bacterial metabolism.

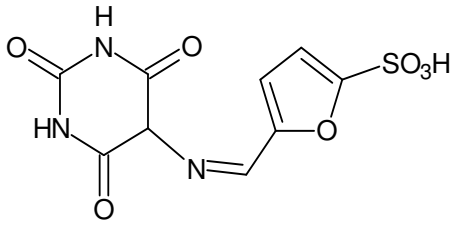
- *Mycobacterium* and *Corneybacterium* species contain ThyA and ThyX genes buy due to poorly understood why both are maintained in these organisms.

The designed drugs are to inhibit the ThyX protein which is ten times more active in the synthesis of DNA than the thyA protein.

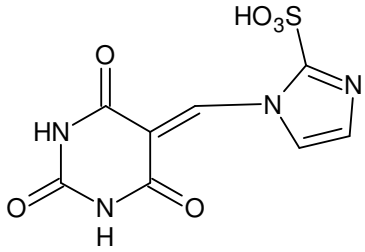
Final synthesized compounds



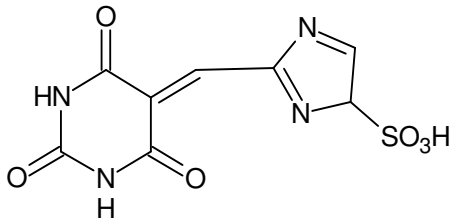
5-[(2,4,6-trioxotetrahydropyrimidin-5(2*H*)-ylidene)methyl]furan-2-sulfonic acid



5-{(*Z*)-[(2,4,6-trioxohexahydropyrimidin-5-yl)imino]methyl}furan-2-sulfonic acid



1-[(2,4,6-trioxotetrahydropyrimidin-5(2*H*)-ylidene)methyl]-1*H*-imidazole-2-sulfonic acid



2-[(2,4,6-trioxotetrahydropyrimidin-5(2*H*)-ylidene)methyl]-4*H*-imidazole-4-sulfonic acid

LIPINSKI RULE

Drug likeness or the property of a chemical compound having pharmacological or biological activity in oral form can be evaluated using Lipinski rule or Rule of five. The rule was formulated by Christopher A. Lipinski in 1997, based on the observation that most medication drugs are relatively small and lipophilic molecules. Rule of five and other properties are used to identify compounds that may be more desirable for high throughput screening and for parallel synthesis efforts.

The physical properties of the molecules were calculated using Molinspiration molecule properties calculator. The results are tabled here.

Compound	LogP	Mol.Wt	TPSA	nOHNH	nON	No of rotatable bonds	No of violations
M1	**-3.17**	**286.221**	**150.305**	**3**	**9**	**2**	**0**
M2	**-3.764**	**301.236**	**155.141**	**3**	**10**	**3**	**0**
M3	**-4.145**	**286.225**	**154.991**	**3**	**10**	**2**	**0**
M4	**-4.288**	**286.225**	**165.848**	**4**	**10**	**2**	**0**

LogP – Partition coefficient

TPSA – Total Polar Surface Area

nOHNH – Number of hydrogen donors

nON – Number of hydrogen acceptors

4.3 Synthesis and characterization

4.3.1 SYNTHESIS [24-27, 43, 44,]

All the compounds are synthesized by microwave technique with a domestic microwave oven.

Knoevenagel condensation

Knoevenagel condensation is condensation of aromatic and heteroaromatic aldehydes with active methylene compounds like barbituric acids, malononitrile, ethyl cyanoacetamide, ethyl cyanoacetate, Meldrum's acid, dimedone and pyrazolone proceeds smoothly with stirring in an aqueous medium. The reactions occur at room temperature giving excellent yields of the products.

Mannich Reaction

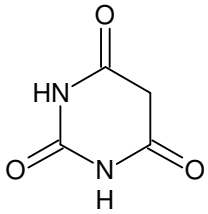
The Mannich reaction is an organic reaction which consists of an amino alkylation of an acidic proton placed next to a carbonyl functional group with formaldehyde and ammonia or any primary or secondary amine. The final product is a β-amino-carbonyl compound also known as a Mannich base

Sulphonation reaction

Sulphonation, is a method by which sulfonic acids are prepared. Important sulfonation procedures include the reaction of aromatic hydrocarbons with sulfuric acid, sulfur trioxide, or chlorosulfuric acid, the reaction of organic halogen compounds with inorganic sulfites; and the oxidation of certain classes of organic sulfur compounds, particularly thiols or disulfides.

REACTANT PROFILE

Barbituric acid;



Synonym **:** melonyl urea, 2,4,6-trioxo hexahydro pyrimidine,

Molecular Formula **:** $C_4H_4N_2O_3$,

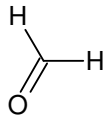
Molecular weight **:** 128.09,

Melting Point **:** 248^oC,

Solubility **:** freely soluble in hot water, Dil.acids, Forms salts with metals,

Nature **:** rhomphus solid,

Formaldehyde



Synonym **:** methylene oxide, formic aldehyde

Molecular Formula **:** CH_2O

Molecular weight **:** 30.03,

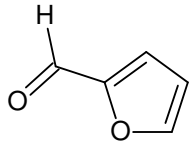
Melting Point **:** -92^oC

Boiling point **:** -19.5^oC

Solubility **:** very soluble in water upto 55%, soluble in alcohol, Ether,

Nature **:** Liquid

Furfural



Synonym **:** 2-Furan carboxaldehyde, 2-Furaldehyde

Molecular Formula **:** $C_5H_4O_2$

Molecular weight **:** 92.8

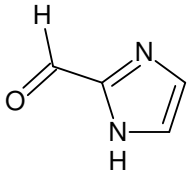
Melting Point **:** -36.5^oC

Boiling point **:** 161.8^oC

Solubility **:** very soluble alcohol, Ether,

Nature **:** colourless oily Liquid, Peculiar odour(Resembling benzaldehyde)

Imidazole;



Synonym **:** Glyoxaline, 1,3-diazole,

Molecular Formula **:** $C_4N_2H_5O$

Molecular weight **:** 87.02

Melting Point **:** 90-91^oC,

Solubility **:** freely soluble water, alcohol, ether, chloroform, pyridine,

Slightly soluble in Pet ether,

Nature **:** stout prisms,

Sulphuric acid

Synonym **:** oil of vitriol,

Molecular Formula **:** H_2SO_4

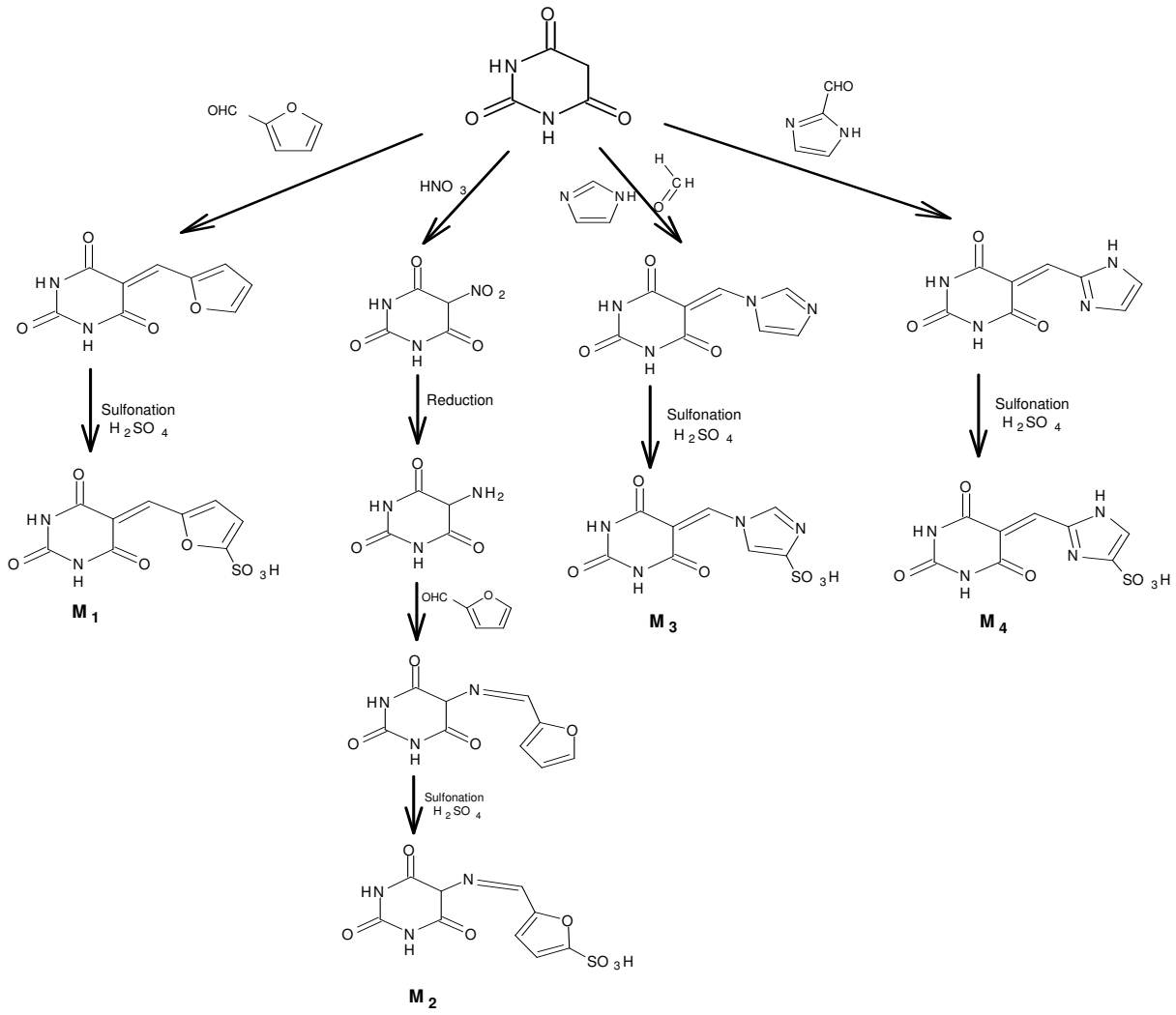
Molecular weight **:** 98.08

Boiling Point **:** 290^oC,

Solubility **:** freely soluble water.

Nature **:** colourless or slightly coloured viscous liquid, emitting chocking fumes of sulphur trioxide.

SYNTHESIS



Synthesis of compound M_1

Barbituric acid (0.007 M), Furfural (0.009 M), was taken in 250ml beaker. The reaction mixture was irradiated on microwave oven at 120^{o}C for 10 mins and then ice cold water added and filtered. The completion of the reaction is confirmed by the disappearance of reactants (monitored by TLC). To the obtained product 2-3 ml of sulphuric acid was added and once again irradiated on microwave oven at 160^{o}C for 5 mins. With the obtained mixture ice cold water was added and filtered and washed with methanol to obtain the product M_1.

Synthesis of compound M_2

Barbituric acid (0.007 M) and Nitric acid (0.009 M) were taken in 250ml beaker and irradiated on microwave oven at 150^{o}C for 10 mins. The obtained product Nitro barbituric acid reduced to amino barbituric acid heating by microwave irradiation and irradiation is continued until there is no yellow color in the liquid. The product washed with hydrochloric acid and then with water.

Amino barbituric acid and furfural (0.009 M) was taken in a 250ml beaker and irradiated with microwave at 120^{o}C for 10 min and then ice cold water added and filtered. The completion of the reaction is confirmed by the disappearance of reactants (monitored by TLC). To the obtained product 2-3 ml of sulphuric acid was added and once again irradiated on microwave oven at 160^{o}C for 10 mins. With the obtained mixture ice cold water was added and filtered and washed with methanol to obtain the product compound M_2.

Synthesis of compound M_3

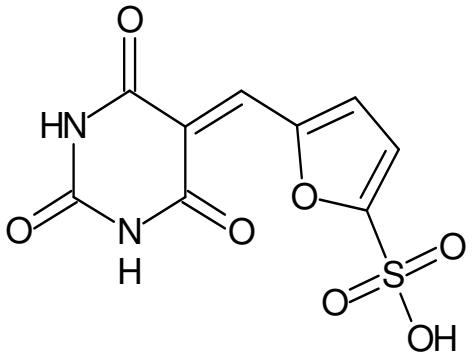
Barbituric acid (0.007 M), Imidazole(0.009 M) and Formaldehyde (0.009 M) are taken in a beaker stirred for 30min at room temperature. The reaction mixture was irradiated on microwave oven at 120^{o}C for 10 mins and then ice cold water added and filtered. The completion of the reaction is confirmed by the disappearance of reactants (monitored by TLC). To the obtained product 2-3 ml of sulphuric acid was added and once again irradiated on microwave oven at 160^{o}C for 5 mins. With the obtained mixture ice cold water was added and filtered and washed with methanol to obtain the product compound M_3.

Synthesis of compound M_4

Barbituric acid (0.007 M) and Immidazole-2-aldehyde (0.009 M) were taken in 250ml beaker. The reaction mixture was irradiated on microwave oven at 120^{o}C for 10mins and then ice cold water added and filtered. The completion of the reaction is confirmed by the disappearance of reactants (monitored by TLC). To the obtained product 2-3 ml of sulphuric acid was added and once again irradiated on microwave oven at 160^{o}C for 5 mins. The obtained mixture was filtered and washed with methanol to obtain the product compound M_4.

4.3.2 CHARACTERIZATION

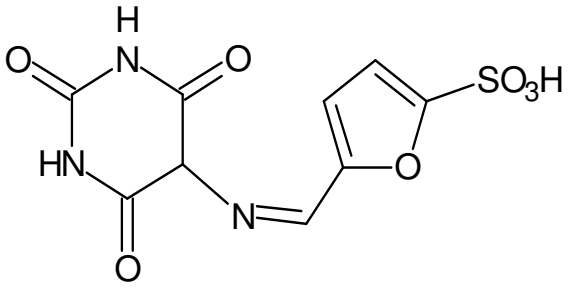
COMPOUND M_1



5-[(2,4,6-trioxotetrahydropyrimidin-5(2*H*)-ylidene)methyl]furan-2-sulfonic acid

Molecular Formula	= $C_9H_6N_2O_7S$
Formula Weight	= 286.21814
Composition	= C(37.77%) H(2.11%) N(9.79%) O(39.13%) S(11.20%)
Molar Refractivity	= 58.02 ± 0.4 cm^3
Molar Volume	= 161.3 ± 3.0 cm^3
Parachor	= 480.4 ± 6.0 cm^3
Index of Refraction	= 1.638 ± 0.02
Surface Tension	= 78.6 ± 3.0 dyne/cm
Density	= 1.773 ± 0.06 g/cm^3
Dielectric Constant	= Not available
Polarizability	= 23.00 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass	= 285.989571 Da
Nominal Mass	= 286 Da
Average Mass	= 286.2181 Da

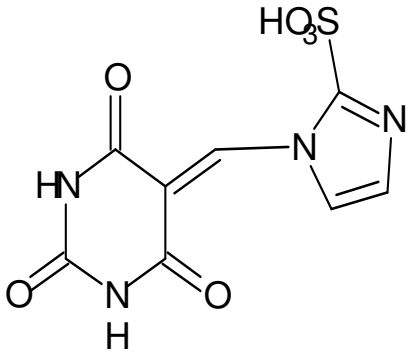
COMPOUND M_2



5-{(*Z*)-[(2,4,6-trioxohexahydropyrimidin-5-yl)imino]methyl}furan-2-sulfonic acid

Molecular Formula	= $C_9H_7N_3O_7S$
Formula Weight	= 301.23278
Composition	= C(35.88%) H(2.34%) N(13.95%) O(37.18%) S(10.64%)
Molar Refractivity	= 62.63 ± 0.5 cm^3
Molar Volume	= 150.6 ± 7.0 cm^3
Parachor	= 474.5 ± 8.0 cm^3
Index of Refraction	= 1.770 ± 0.05
Surface Tension	= 98.5 ± 7.0 dyne/cm
Density	= 2.00 ± 0.1 g/cm^3
Dielectric Constant	= Not available
Polarizability	= 24.83 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass	= 301.00047 Da
Nominal Mass	= 301 Da
Average Mass	= 301.2328 Da

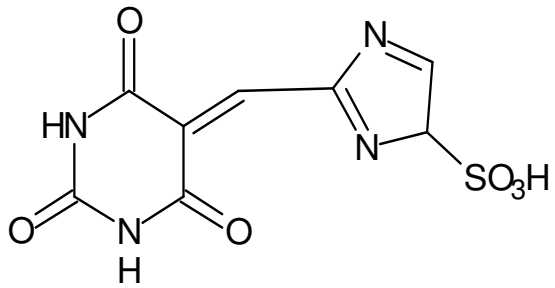
COMPOUND M_3



1-[(2,4,6-trioxotetrahydropyrimidin-5(2*H*)-ylidene)methyl]-1*H*-imidazole-2-sulfonic acid

Molecular Formula	= $C_8H_6N_4O_6S$
Formula Weight	= 286.22144
Composition	= C(33.57%) H(2.11%) N(19.57%) O(33.54%) S(11.20%)
Molar Refractivity	= 60.59 ± 0.5 cm^3
Molar Volume	= 138.8 ± 7.0 cm^3
Parachor	= 450.8 ± 8.0 cm^3
Index of Refraction	= 1.823 ± 0.05
Surface Tension	= 111.2 ± 7.0 dyne/cm
Density	= 2.06 ± 0.1 g/cm^3
Dielectric Constant	= Not available
Polarizability	= 24.02 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass	= 286.000804 Da
Nominal Mass	= 286 Da
Average Mass	= 286.2214 Da

COMPOUND M_4



2-[(2,4,6-trioxotetrahydropyrimidin-5(2*H*)-ylidene)methyl]-4*H*-imidazole-4-sulfonic acid

Molecular Formula	= $C_8H_6N_4O_6S$
Formula Weight	= 286.22144
Composition	= C(33.57%) H(2.11%) N(19.57%) O(33.54%) S(11.20%)
Molar Refractivity	= 59.69 ± 0.5 cm^3
Molar Volume	= 132.7 ± 7.0 cm^3
Parachor	= 436.4 ± 8.0 cm^3
Index of Refraction	= 1.857 ± 0.05
Surface Tension	= 116.9 ± 7.0 dyne/cm
Density	= 2.15 ± 0.1 g/cm^3
Dielectric Constant	= Not available
Polarizability	= 23.66 ± 0.5 $10^{-24}cm^3$
Monoisotopic Mass	= 286.000804 Da
Nominal Mass	= 286 Da
Average Mass	= 286.2214 Da

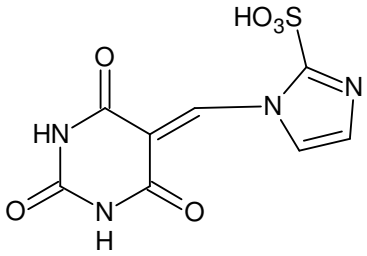
CHARACTERIZATION [45- 48]

The synthesized compounds were characterized using following experimental methods.

1. **Infrared spectroscopy (IR) by Perkin-Elmer Spectrometer using KBr pellets.**
2. **Nuclear Magnetic Resonance spectroscopy (^{1}H NMR) by 500 MHZ Jeol using DMSO.**
3. **Mass spectroscopic (MS) by Jeol GC mate.**
4. **Melting Point by One side open ended capillary tubes.**

ANALYTICAL DATA

COMPOUND M_1



1-[(2,4,6-trioxotetrahydropyrimidin-5(2*H*)-ylidene)methyl]-1*H*-imidazole-2-sulfonic acid

S.No	Properties	
1	Molecular formula	$C_9H_6N_2O_7S$
2	Molecular weight	285
3	Description	Black shiny solid
4	Melting point	284 ^oC
5	Percentage yield	83%

IR υ^{cm-1} (KBr):

3100-3000cm^{-1} (C-H), 2854 cm^{-1}(O-H), 1713cm^{-1} (C=C), 1270-1020cm^{-1} (C-C), 1663cm^{-1}(C=O), 910cm^{-1}(S-O), 671cm^{-1}(C-S), 3425cm^{-1}(N-H), 1070cm^{-1}(C-N).

^{1}H NMR Data (500 MHz, DMSO-d_6)

δ 2.0 (s, 1H, -OH), 6.2 (s, 1H, Hetero aromatic C-H), 7.4 (s, 1H,Hetero aromatic C-H), 8.01 (s, 1H, -CH), 10.0 (s, 2H, N-H).

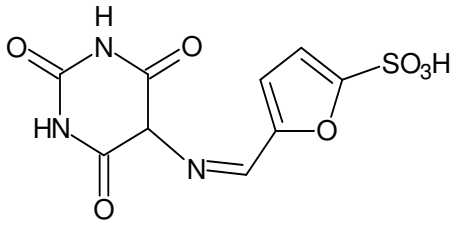
^{13}C NMR Data (500 MHz, DMSO-d_6)

δ 39.493,39.660, 39.828, 39.995, 40.162, 40.325, 40.495, 67.102, 84.659.

MASS (m/e value)

285.99(**M^+**) (9%), 62.16(**B**) (100%).

COMPOUND M_2



5-{(*Z*)-[(2,4,6-trioxohexahydropyrimidin-5-yl)imino]methyl}furan-2-sulfonic acid

S.No	Properties	
1	Molecular formula	$C_9H_7N_3O_7S$
2	Molecular weight	301
3	Description	Grey fine powder
4	Melting point	276 ^oC
5	Percentage yield	76%

IR υ^{cm-1} (KBr):

1651cm^{-1} (C=O), 3448cm^{-1}(N-H), 1142cm^{-1}(C-N), 1481cm^{-1}(C-C), 1450cm^{-1}(C=N), 3024cm^{-1}(C-H), 1296cm^{-1}(C-O), 2900cm^{-1}(O-H), 856cm^{-1}(S-O), 694 cm^{-1}(C-S).

^{1}H NMR Data (500 MHz, DMSO-d_6)

δ 2.0 (s, 1H, -OH), 3.3 (s, 1H, C-H), 7.3 (s, 1H, Aldimine-CH), 6.2 (s, 2H, ,Hetero aromatic CH), 10.0 (s, 2H, N-H).

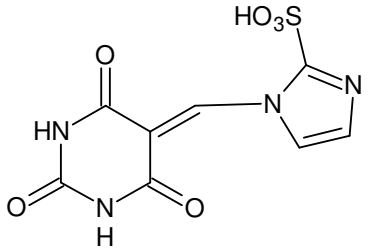
^{13}C NMR Data (500 MHz, DMSO-d_6)

δ 39.623, 39.789, 39.956, 40.123, 40.290, 113.235, 150.257, 159.709.

MASS (m/e value)

301.28(**M^+**) (7%), 61.78(**B**) (100%).

COMPOUND M_3



1-[(2,4,6-trioxotetrahydropyrimidin-5(2*H*)-ylidene)methyl]-1*H*-imidazole-2-sulfonic acid

S.No	Properties	
1	Molecular formula	$C_8H_6N_4O_6S$
2	Molecular weight	386
3	Description	Yellow coarse power
4	Melting point	265 ^oC
5	Percentage yield	83%

IR υ^{cm-1} (KBr):

1690cm^{-1} (C=O), 3418cm^{-1}(N-H), 1088cm^{-1}(C-N), 1489cm^{-1}(C-C), 1412cm^{-1}(C=N), 779cm^{-1}(C-C), 3047cm^{-1}(C-H), 2939cm^{-1}(O-H), 895cm^{-1}(S-O).

^{1}H NMR Data (500 MHz, DMSO-d_6)

δ 2.5 (s, 1H, -OH), 6.6 (s, 1H, C-H), 7.1 (s, 2H, ,Hetero aromatic C-H), 10.5 (s, 2H, N-H).

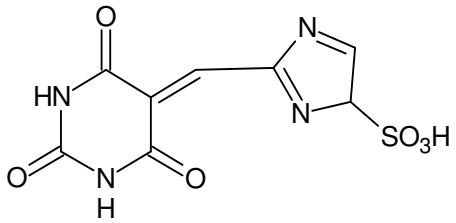
^{13}C NMR Data (500 MHz, DMSO-d_6)

δ 141.249, 141.332, 152.101, 154.026, 156.149, 158.201.

MASS (m/e value)

286.21(**M^+**) (4%), 65.46(**B**) (100%).

COMPOUND M_4



2-[(2,4,6-trioxotetrahydropyrimidin-5(2*H*)-ylidene)methyl]-4*H*-imidazole-4-sulfonic acid

S.No	Properties	
1	Molecular formula	$C_8H_6N_4O_6S$
2	Molecular weight	386
3	Description	Sandy yellow fine power
4	Melting point	272 ^oC
5	Percentage yield	52%

IR υ^{cm-1} (KBr):

1690cm^{-1} (C=O), 3418cm^{-1}(N-H), 1088cm^{-1}(C-N), 1489cm^{-1}(C-C), 1412cm^{-1}(C=N), 779cm^{-1}(C-C), 3047cm^{-1}(C-H), 2939cm^{-1}(O-H), 895cm^{-1}(S-O).

^{1}H NMR Data (500 MHz, DMSO-d_6)

δ 2.0 (s, 1H, -OH), 6.6 (s, 1H, C-H), 7.2 (s, 1H, ,Hetero aromatic -CH), 7.7 (s, 1H, Hetero aromatic -CH), 10.3 (s, 2H, N-H).

^{13}C NMR Data (500 MHz, DMSO-d_6)

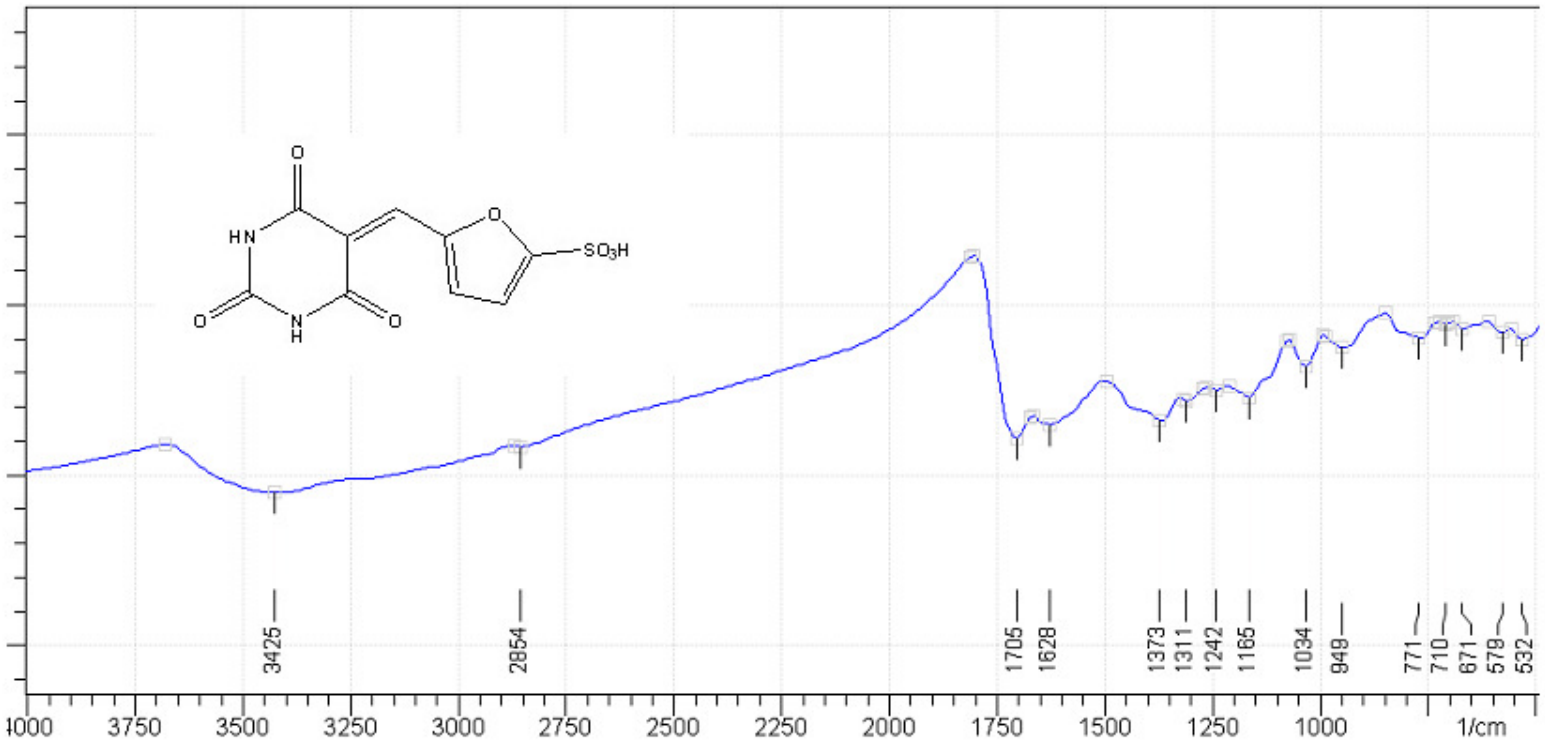
δ 77.795, 150.249, 151.332, 151.101,164.026, 166.149, 168.201.

MASS (m/e value)

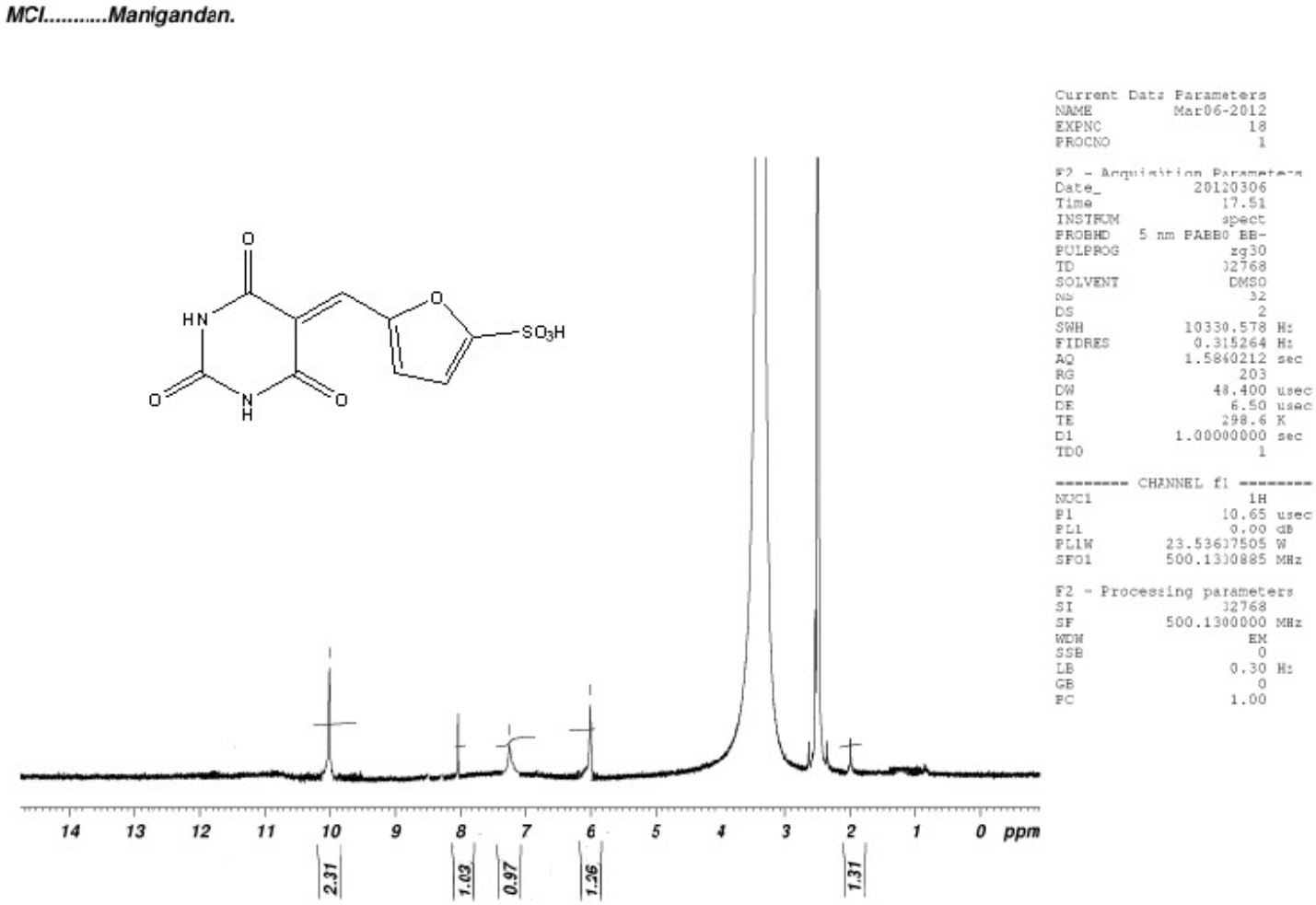
386.21(**M^+**) (3%), 63.34(**B**) (100%).

COMPOUND M_1 5-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl] furan-2-sulfonic acid.

IR SPECTRA

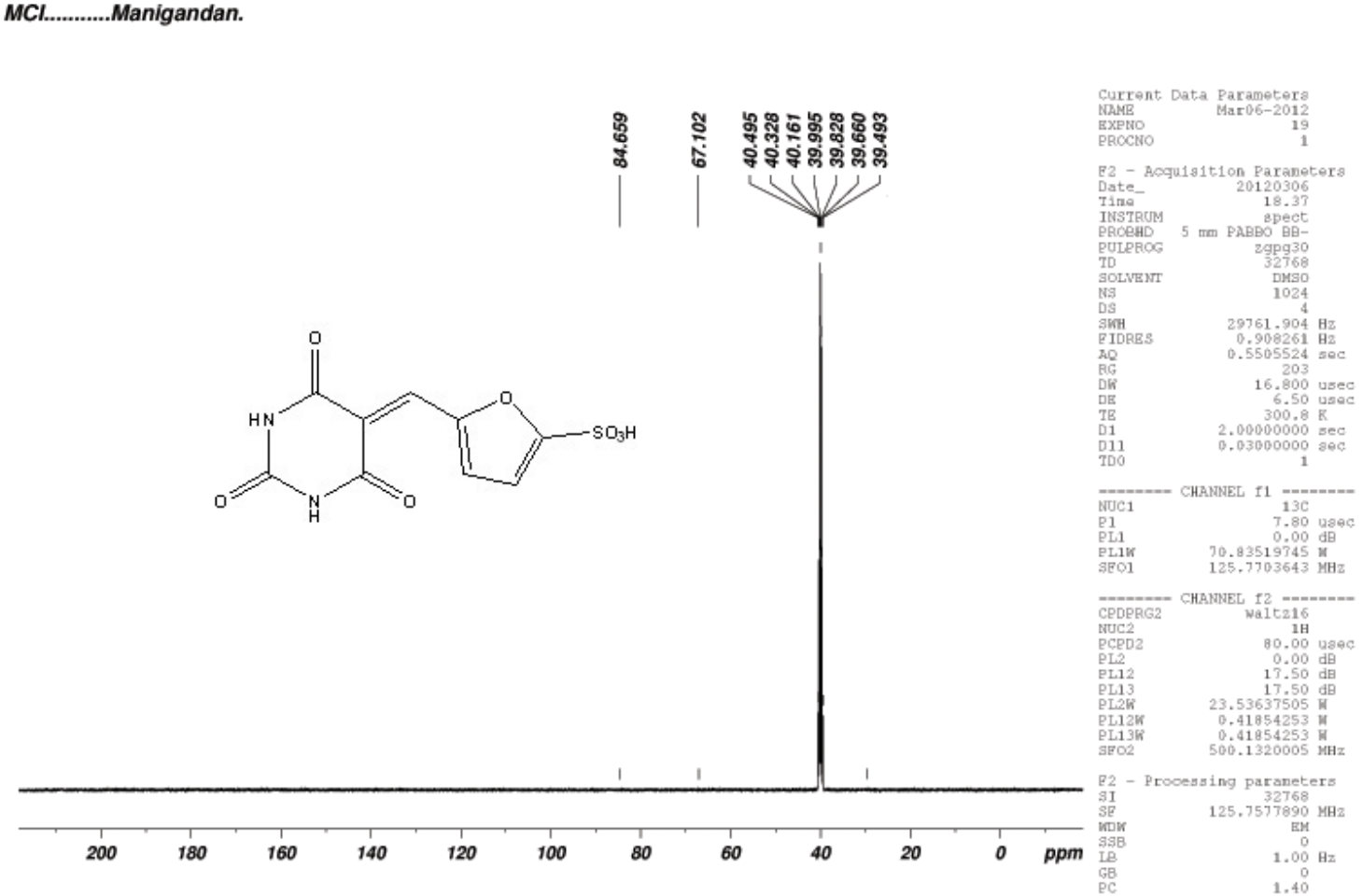


COMPOUND M_1 5-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl] furan-2-sulfonic acid.

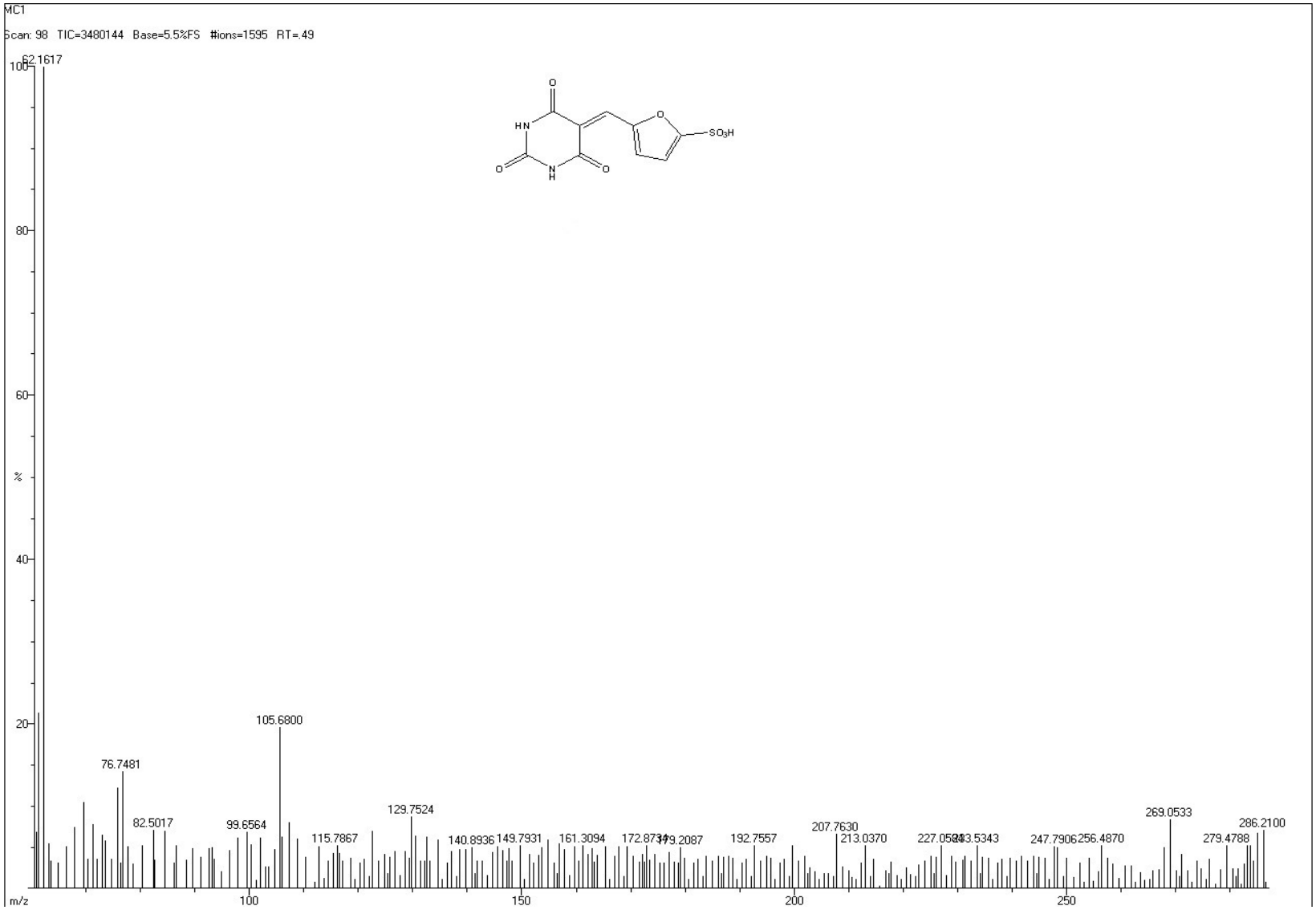


COMPOUND M_1 5-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl] furan-2-sulfonic acid.

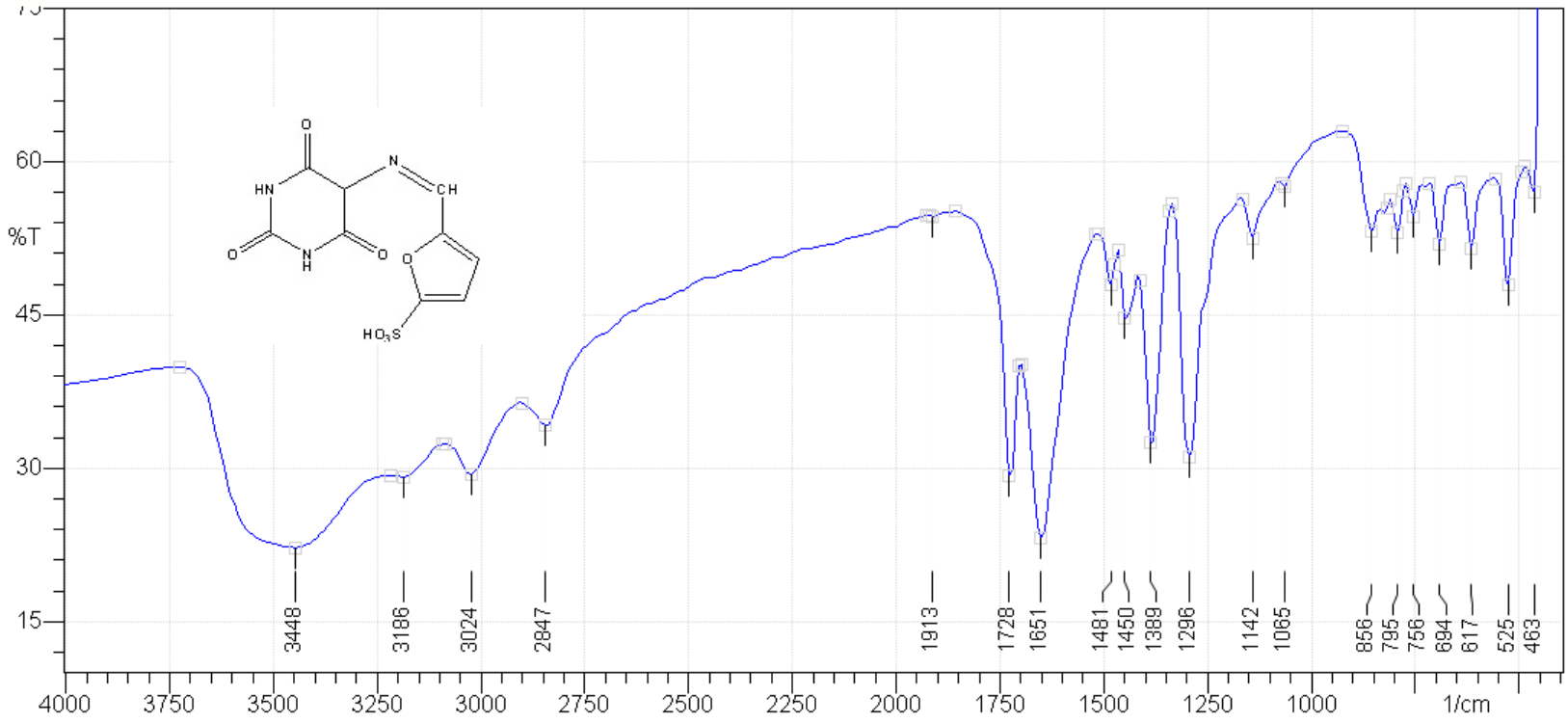
^{13}C NMR SPECTRA



COMPOUND M_1 5-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl] furan-2-sulfonic acid.

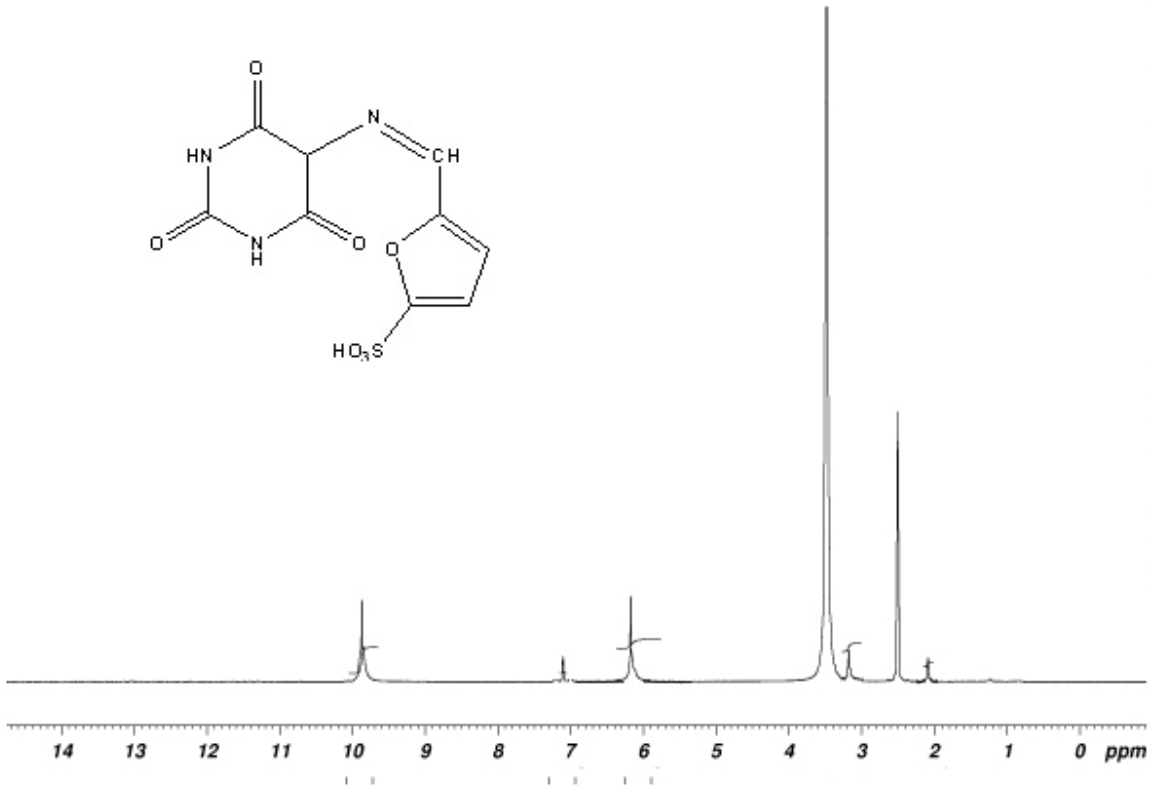


COMPOUND M_2 5-{(*Z*)-[(2, 4, 6-trioxohexahydropyrimidin-5-yl) imino] methyl} furan-2-sulfonic acid.



COMPOUND M_2 5-{(*Z*)-[(2, 4, 6-trioxohexahydropyrimidin-5-yl) imino] methyl} furan-2-sulfonic acid.

MCII.....Manigandan.

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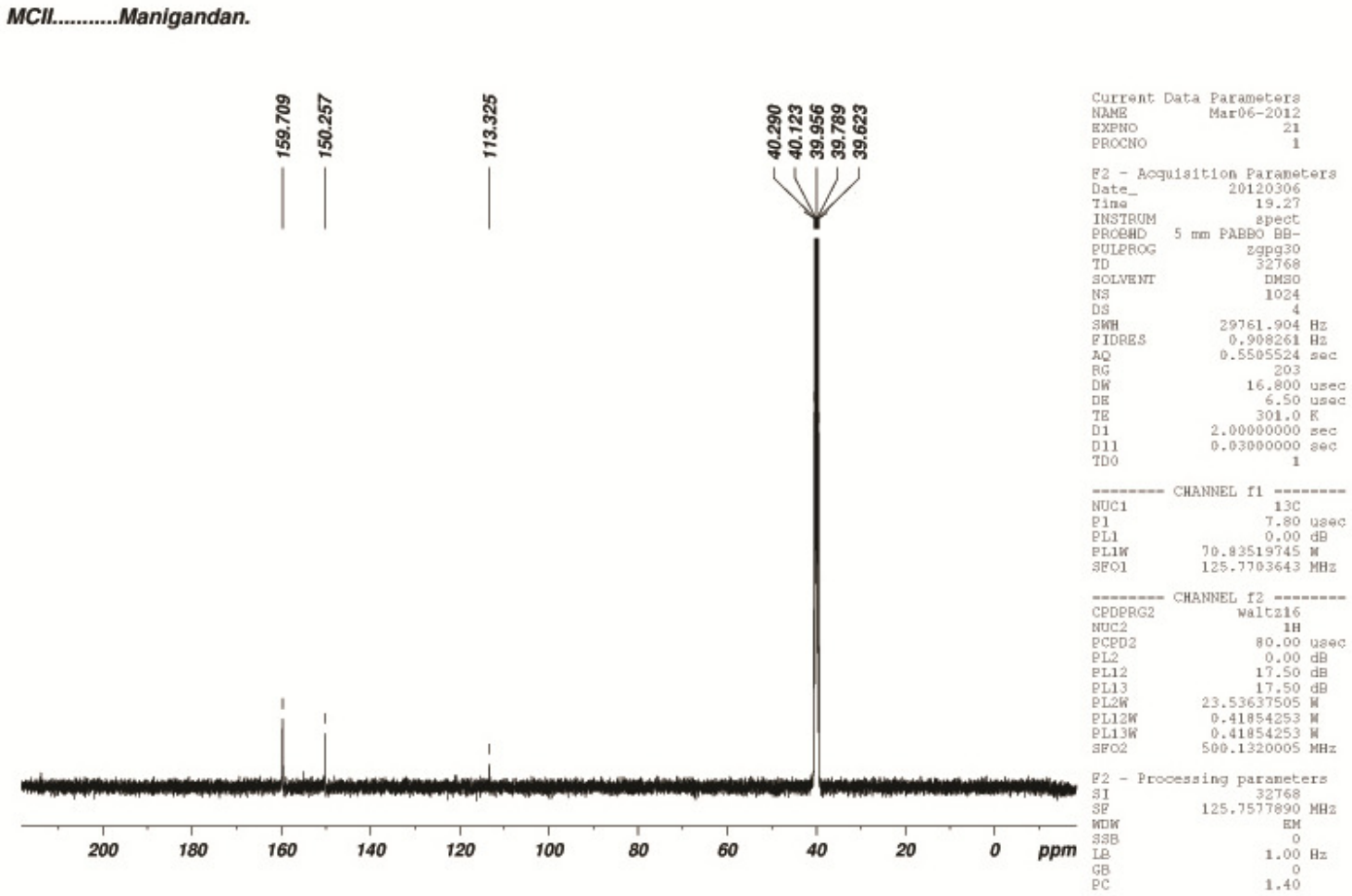
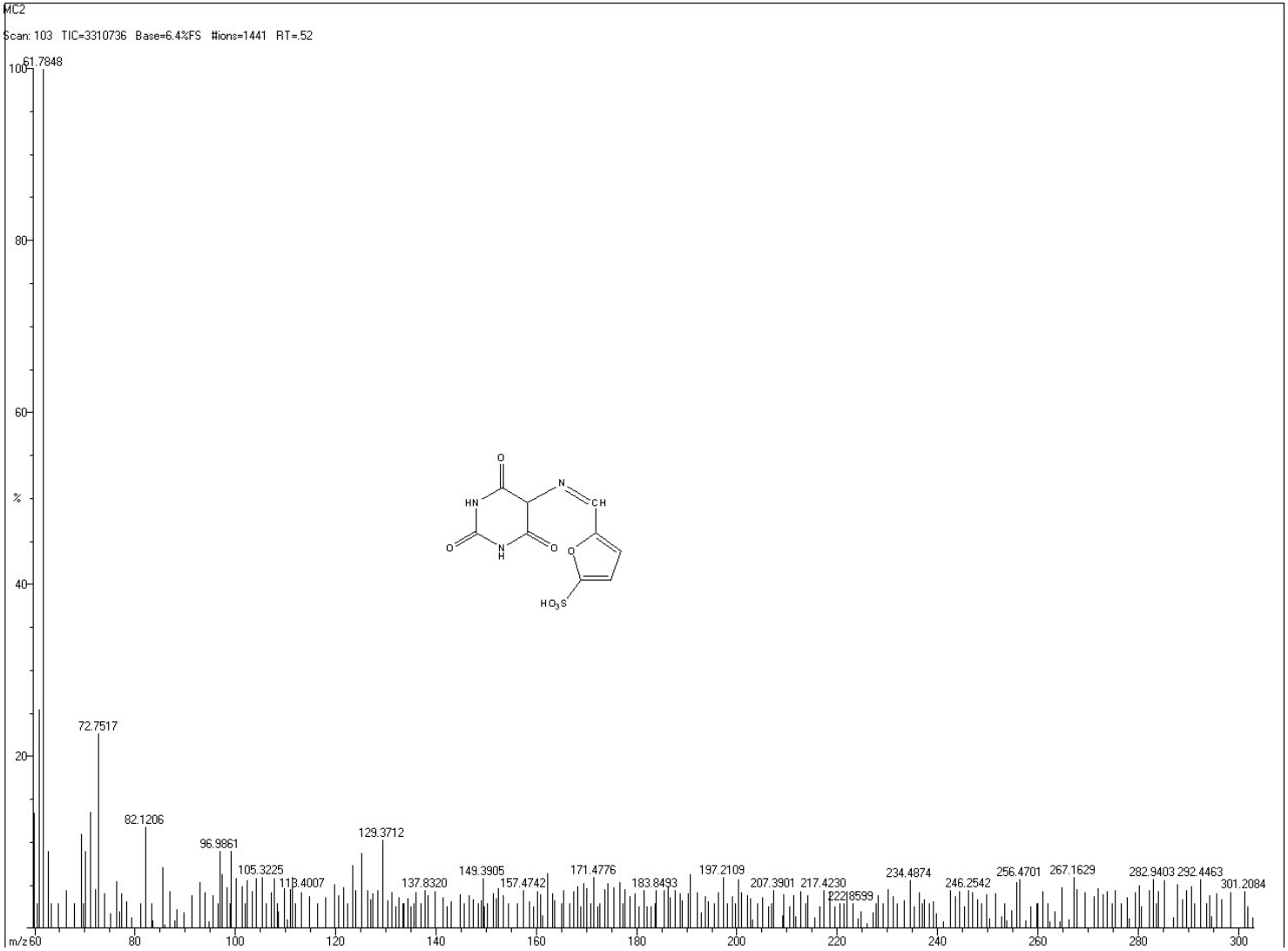
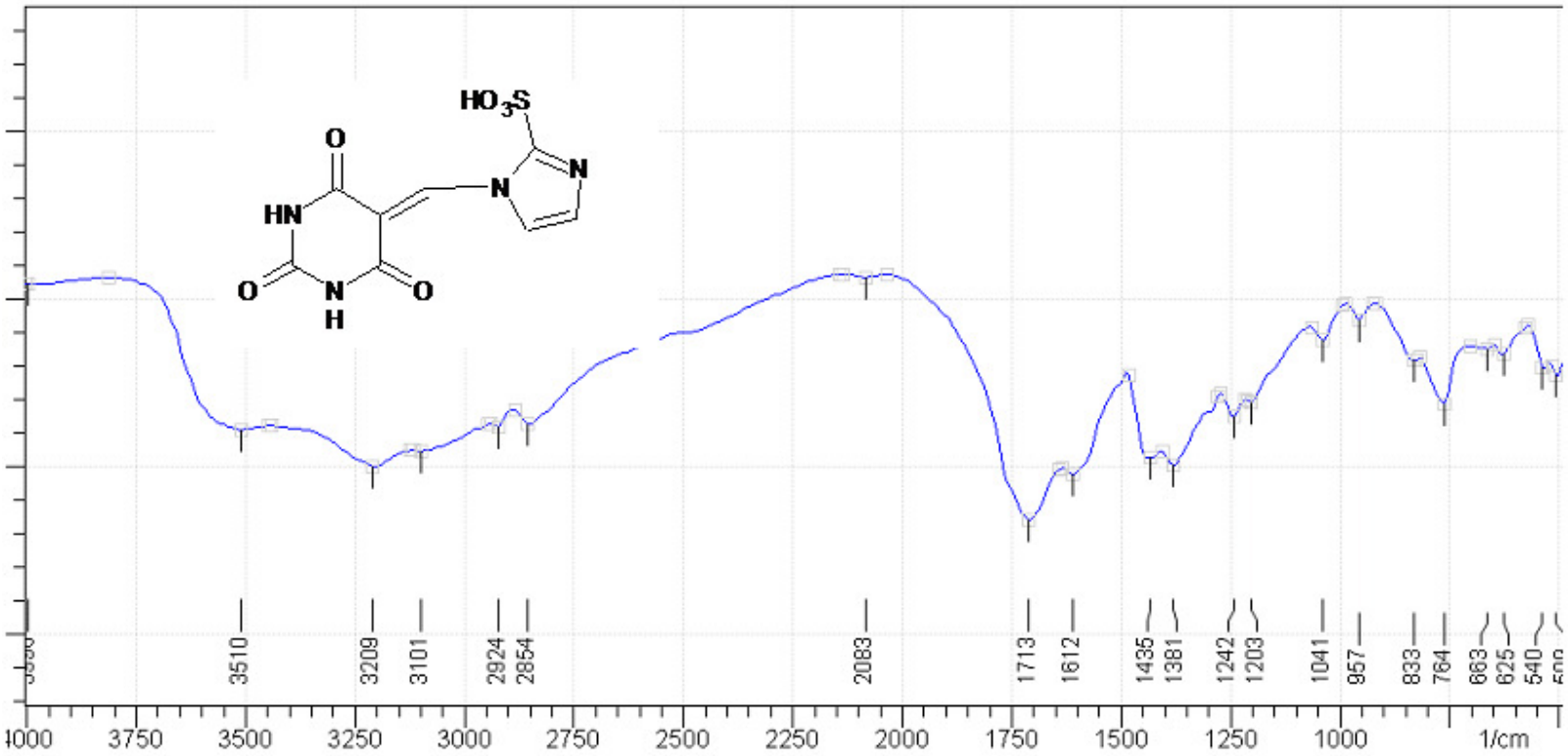
COMPOUND M_2 5-{(*Z*)-[(2, 4, 6-trioxohexahydropyrimidin-5-yl) imino] methyl} furan-2-sulfonic acid.



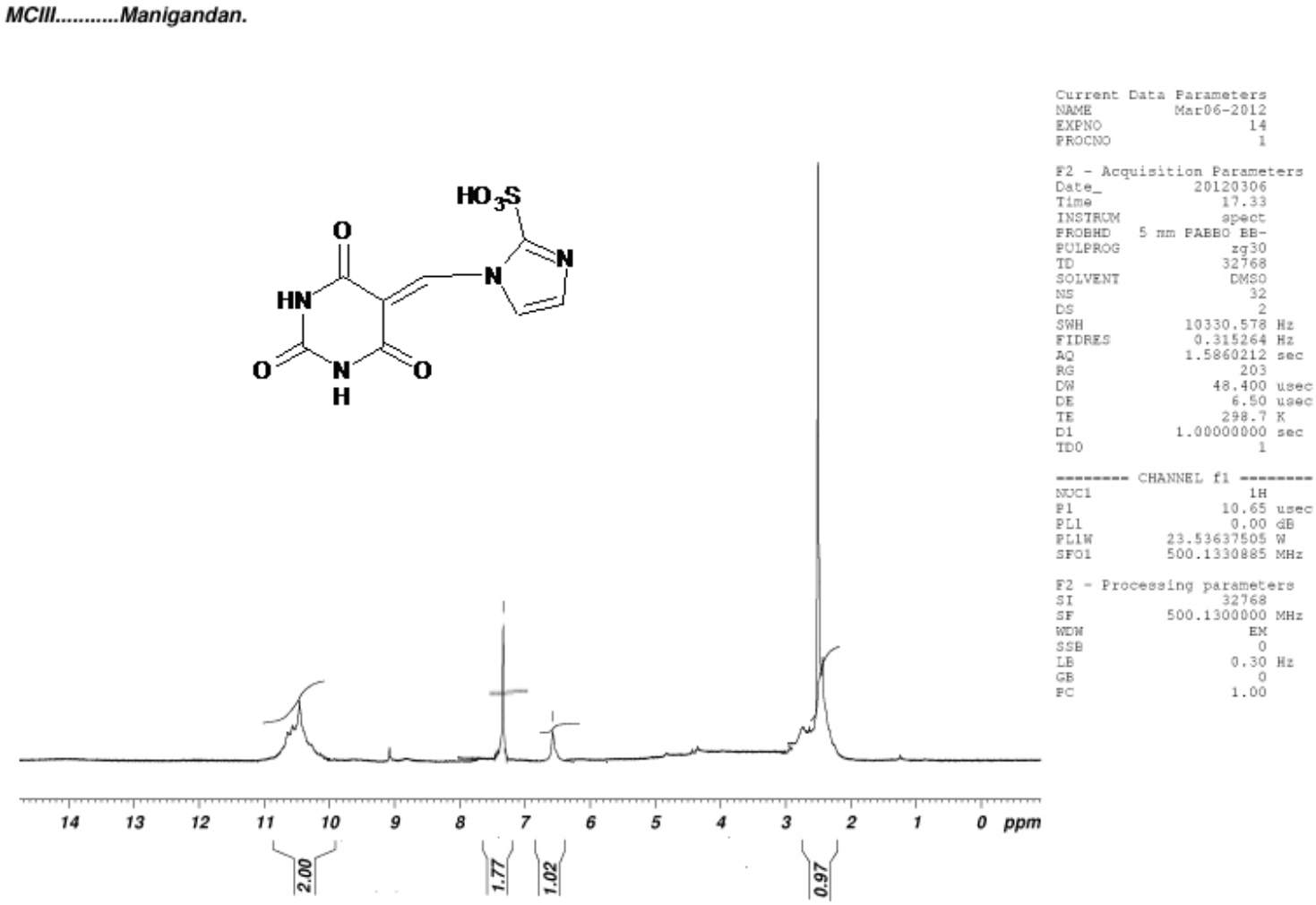
COMPOUND M_2 5-{(*Z*)-[(2, 4, 6-trioxohexahydropyrimidin-5-yl) imino] methyl} furan-2-sulfonic acid.



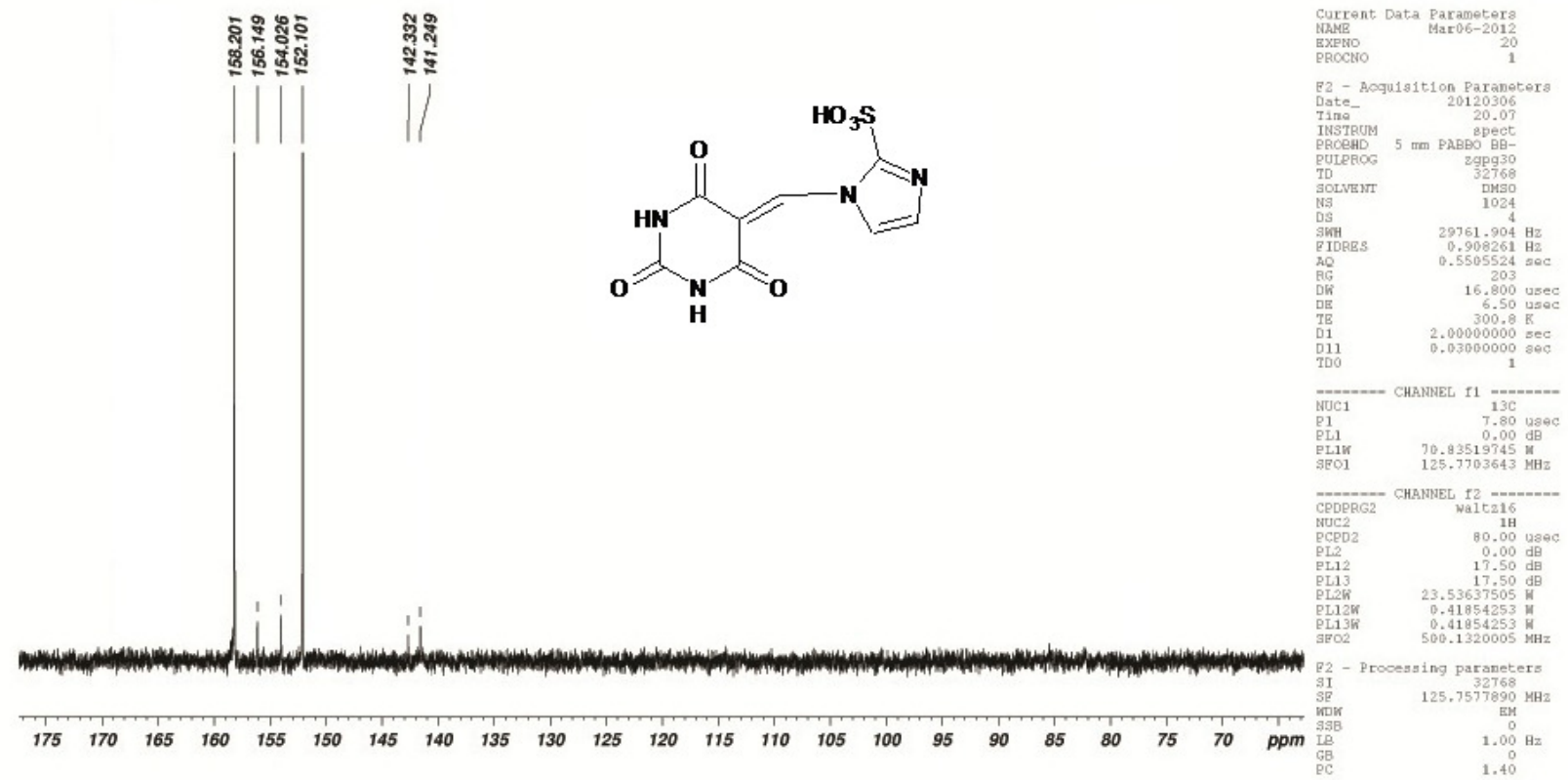
COMPOUND M_3 5-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl] furan-2-sulfonic acid.



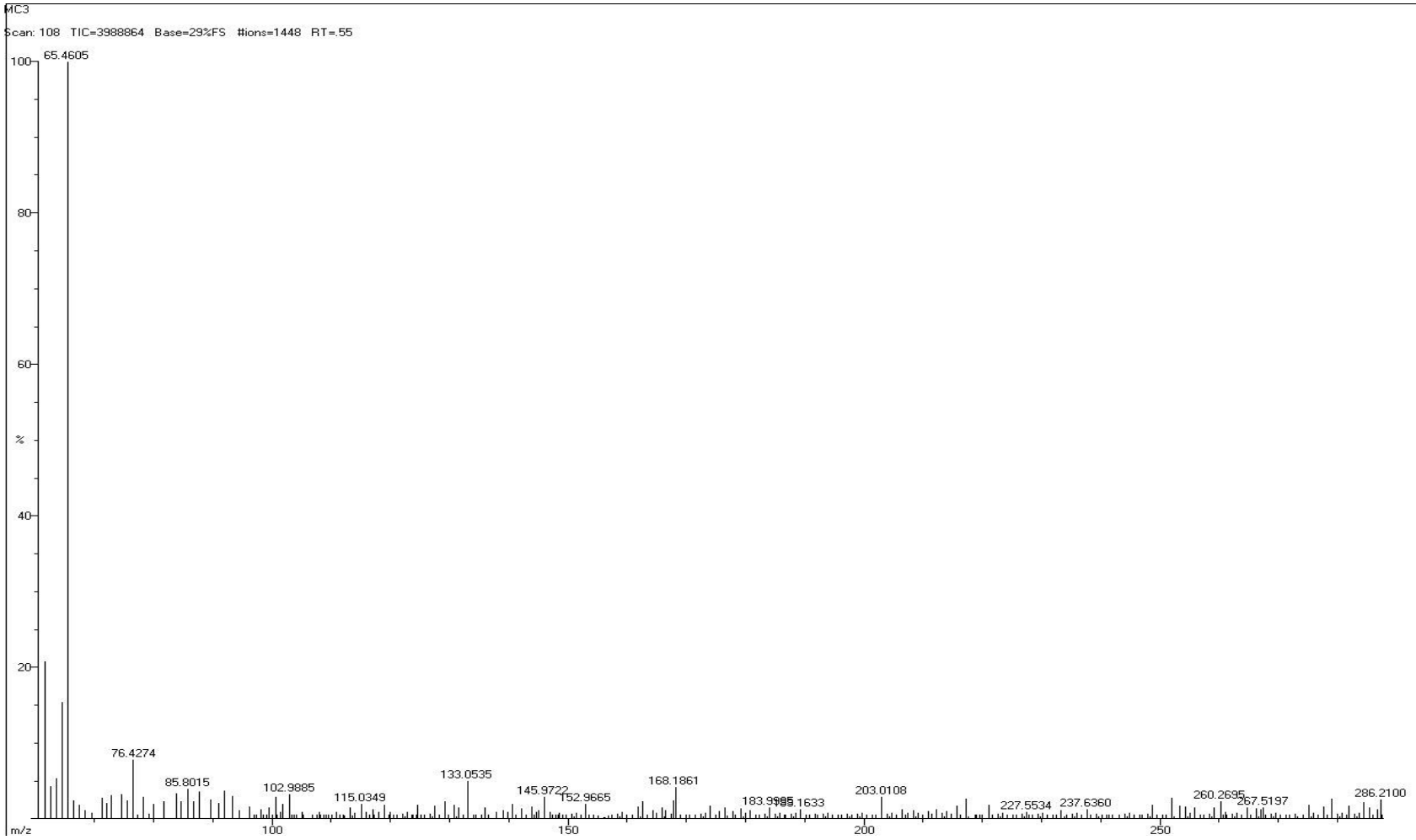
COMPOUND M_3 5-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl] furan-2-sulfonic acid.



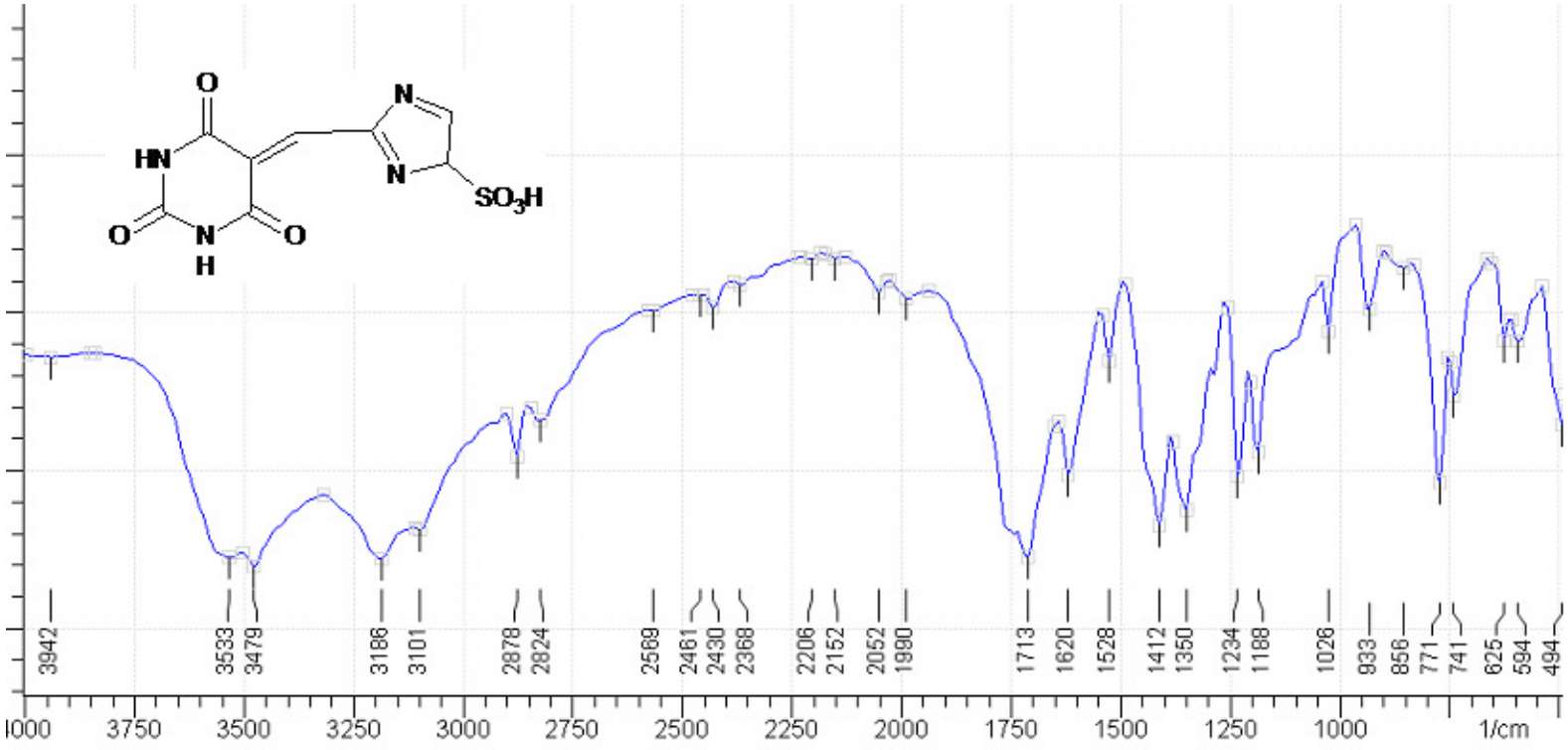
COMPOUND M_3 5-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl] furan-2-sulfonic acid.



COMPOUND M_3 5-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl] furan-2-sulfonic acid.

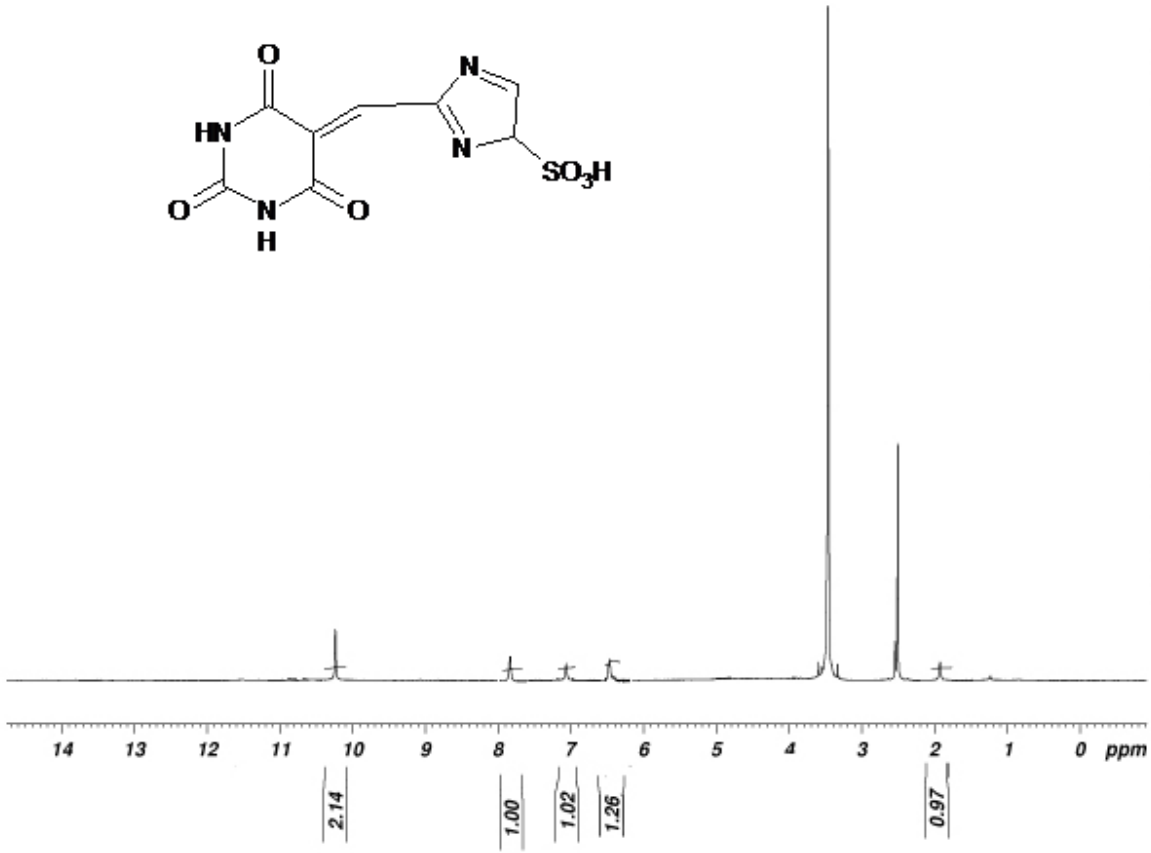


COMPOUND M_4 2-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl]-4*H*-imidazole-4-sulfonic acid.



COMPOUND M_4 2-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl]-4*H*-imidazole-4-sulfonic acid.

MCIV.....Manigandan.

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FIDRES         0.315264 Hz
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D1           1.00000000 sec
TD0                    1

======== CHANNEL f1 ========
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P1                 10.65 usec
PL1                0.00 dB
PL1W       23.53637505 W
SFO1       500.1330885 MHz

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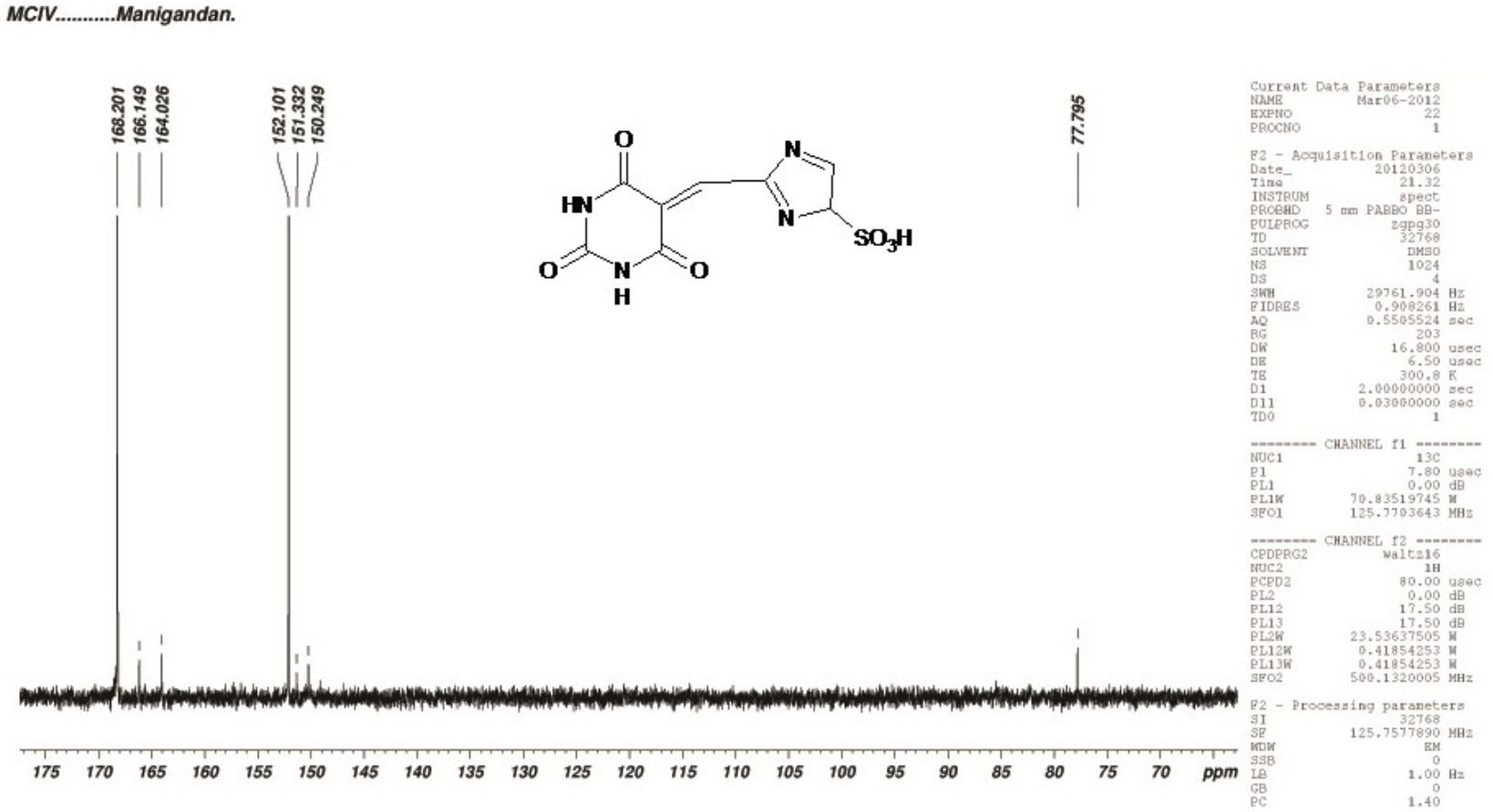
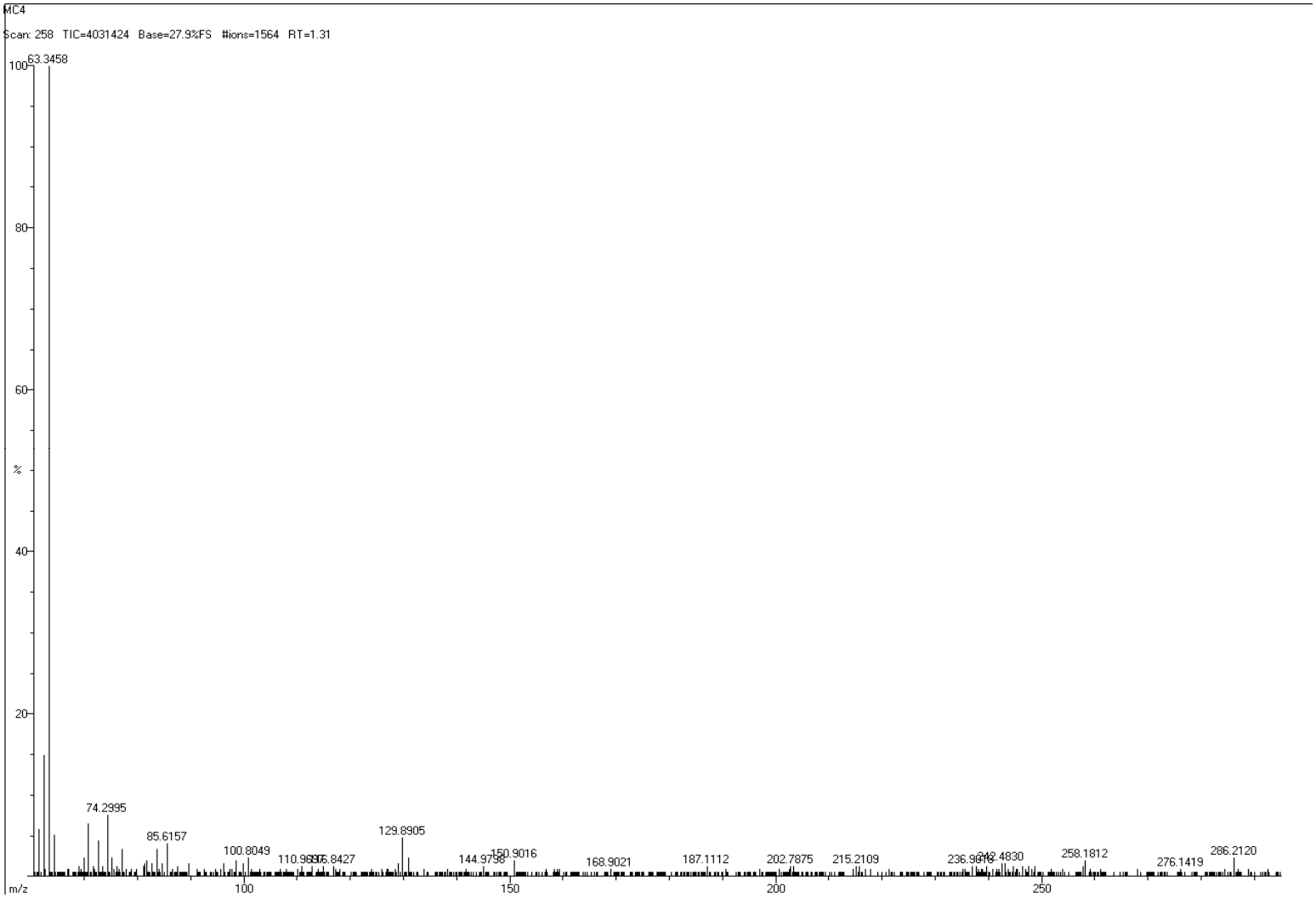
COMPOUND M_4 2-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl]-4*H*-imidazole-4-sulfonic acid.



COMPOUND M_4 2-[(2, 4, 6-trioxotetrahydropyrimidin-5(2*H*)-ylidene) methyl]-4*H*-imidazole-4-sulfonic acid.



INVITRO ANTITUBERCULAR STUDY [32-34]

Drug susceptibility testing of *Mycobacterium tuberculosis* by the Broth Micro-dilution method.

Inoculum preparation

- Freshly grown colonies of H37RV from LJ medium were transferred to a tube containing 3-4 ml PBS and 6 to 9 sterile glass beads.

- Tubes were vigorously agitated on a vortex mixer and clumps were allowed to settle for 30 mins.

- The supernatant were transferred to a sterile McCartney bottle.

- The supernatant were then adjusted with PBS to equal the turbidity of 0.5 Mc Farland standard for use as the standard inoculums in Broth Micro dilution method (BMM).

Turbidity standard for inoculum preparation

- In microbiology, **McFarland standard is** used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria in a specific volume of broth will be within a given range

- The standard can be compared visually to a suspension of bacteria in sterile saline or nutrient broth. If the bacterial suspension is too turbid, it can be diluted with more diluents. If the suspension is less, additional volume of bacterial suspension can be added.

- To standardize the inoculums density for a susceptibility test, a $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standard. A $BaSO_4$ 0.5 McFarland standard may be prepared as follows:

a) A 0.5-ml aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2$. $2H_2O$) is added to 99.5 ml of 0.18 mol/L H_2SO_4 (1% v/v) with constant stirring to maintain a suspension.

b) The correct density of the turbidity standard should be verified by using a spectrophotometer to determine the absorbance. The absorbance at 625 nm should be 0.008 to 0.10 for the 0.5 McFarland standard.

MIC plate's preparation

- The plates used were 96-well microtitre plates (TPP) with U-shaped wells.
- The microwells were filled with 0.1 ml of Middlebrook 7H9 broth, supplemented with ADC enrichment.
- The stock suspension of each test drug (sterilized in 0.22µm Millipore syringe filter) was diluted by adding Middlebrook 7H9 broth and 0.1 ml volume was dispensed into plates and serial dilution for each drug were prepared.
- Each well of the microtiter plate containing 0.1 ml of Middlebrook 7H9 broth and test drug was inoculated with 5 µl of standard bacterial suspension.
- A control well with Middlebrook 7H9 broth was inoculated with 5 µl of standard bacterial suspension as growth control.
- One bacterial suspension free broth was kept as broth control.
- The plates were sealed and incubated at 37º C for 21 days in a incubator
- The growth of the organism was ascertained by visual observation.

- MIC well was identified as the lowest concentration that exhibited no growth by visual reading.

Minimum Bactericidal Concentration (MBC) tests by recovery plate [31]

- This was taken to be the concentration of the test drug that prevented growth by 99 percent or greater, compared with the untreated controls.

- Fresh Middle brook 7H11agar in 90-mm plates with division were then prepared, and inoculated with a loop full of inocula from the wells and the procedure for incubation repeated for up to four weeks.

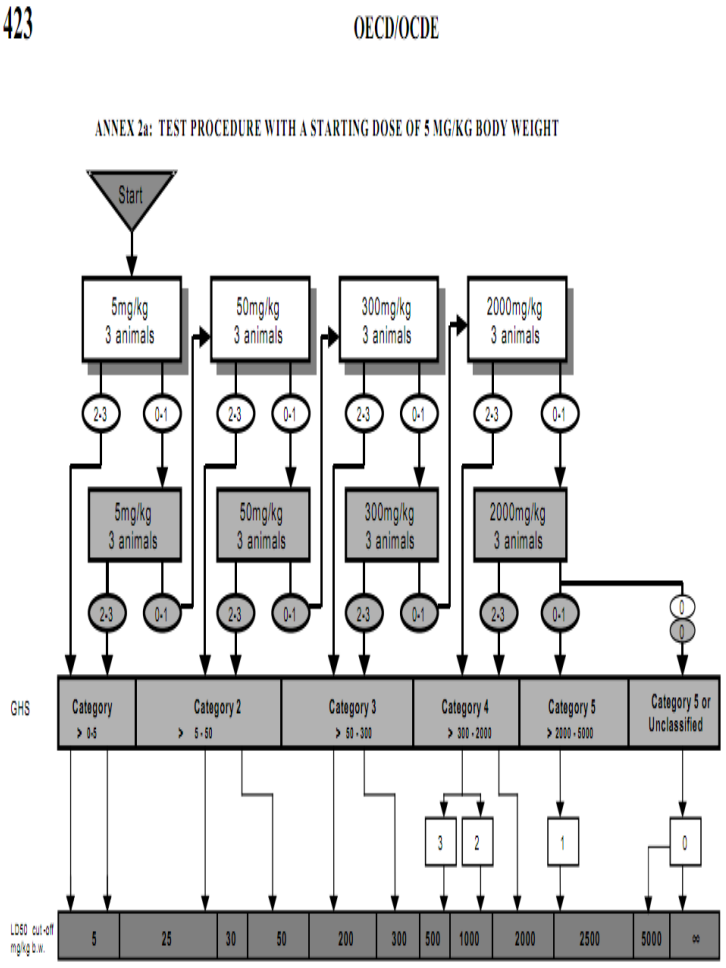
ACUTE TOXICITY STUDY [49]

This acute toxicity study was designed as per OECD guideline for Testing of Chemicals, Acute Oral toxicity (Acute Toxic Class method), Guideline 423

Experimental Procedure

Healthy adult female Wister Albino mice (3 Nos) were used in this test. Females used were weighing between 20-25g, nulliparous and non-pregnant. For all the three animals food, but water was withheld overnight prior to dosing.

The compounds were administered orally to a group of of experimental animals at one of the defined dose (2000mg/kg).



Observation

The animals were observed individually after dosing once during the first 30minutes, periodically for the 24hrs, with special attention giving during the first 4 hrs, and daily there-after, for a total of 14 days. The following clinical observations were made and recorded.

- **Toxic designs:** All the rats were observed for any toxic signs.

- **Pre-terminal deaths**: All the rats were observed for any pre-terminal deaths.

- **Body weight**: Individual body weight was recorded for all the animals once in a week.

- **Cage side observation**: The faeces colour and consistency, changes in skin and fur, eyes and mucous membrane (nasal) of the animal were observed once in a week.

RESULTS

&

DISCUSSION

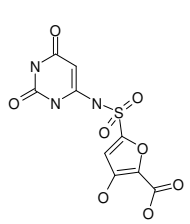
5. Results and Discussion

The results of the Drug design, Synthesis, Characterization, In-vitro evaluation and toxicological study are compiled and presented.

5.1 DRUG DESIGN

Final compounds

Shortlisted compounds after docking, HIPHOP and 2D similarity searching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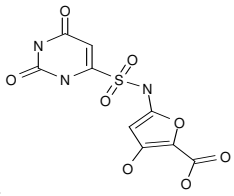
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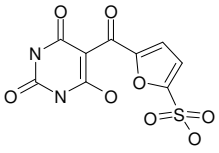
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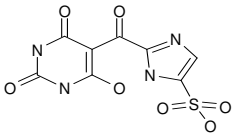
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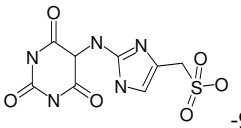
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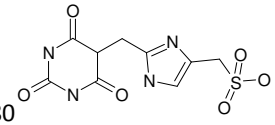
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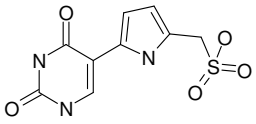
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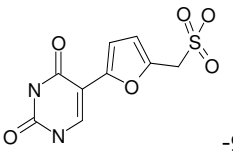
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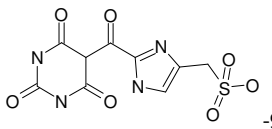
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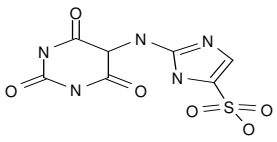
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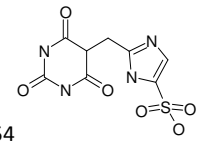
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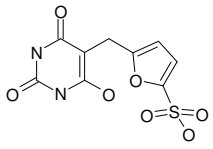
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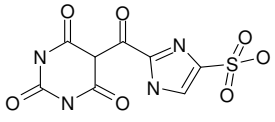
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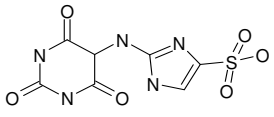
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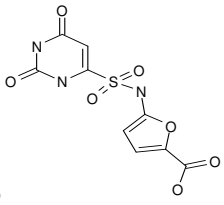

9.23

Structural activity relationship

- ✓ Pyrimidine dione or trione connected with the five membered ring is the minimum requirement for the activity. If the five membered ring or 6 membered ring replaced by other rings or any other changes in the ring leads to loss of activity.

- ✓ Presence of carbonyl group in the 6 membered ring will increase the activity. Pyrimidine dione and trione binds more effectively than the pyrimidinone.

- ✓ Replacement of 5 membered ring with any other ring will lead to loss of activity.

- ✓ The 6 membered and 5 membered ring can be connected through one or two atoms. If they are more than two atoms apart will terminate the activity. Same time it should not be attached like spiro compound.

- ✓ Presence of oxygen containing functional groups in the five membered rings is must for binding to the receptor. Like COOH, OH, SO3H, PO4, etc.

Ligand – Receptor Interaction

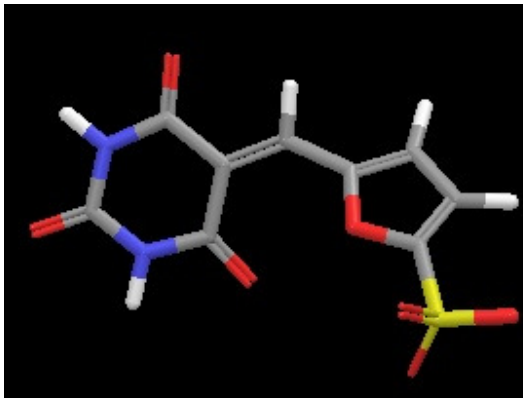
Based on the above SAR four compounds were designed and synthesized and they have best interaction in the binding site of the receptor. All the four molecules have better interaction with the amino acids ARG21, ARG166, SER167, ASP169, HIP 207, and ASN177.

Lipinski rule

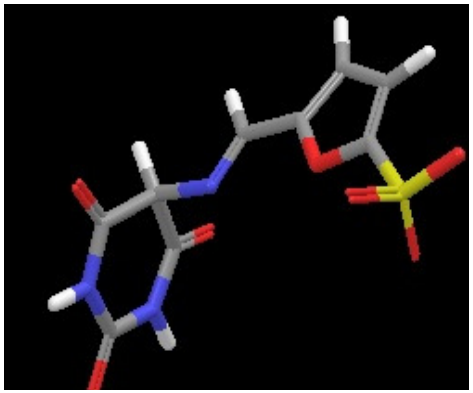
All the compounds pass the rule of five and there is no violation in the basic properties. It proves that our molecule has druglikeness to reach the target site for the biological action. We conclude that our molecules have positive nature on ADME character.

DOCKING SCORE OF SYNTHESISED COMPOUNDS

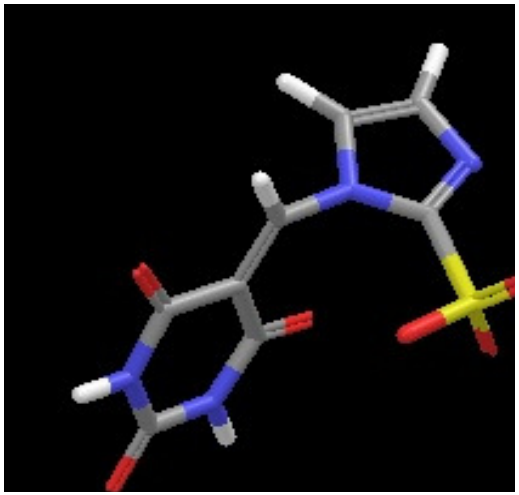
Compound M_1



Compound M_2



Compound M_3



Compound M_4

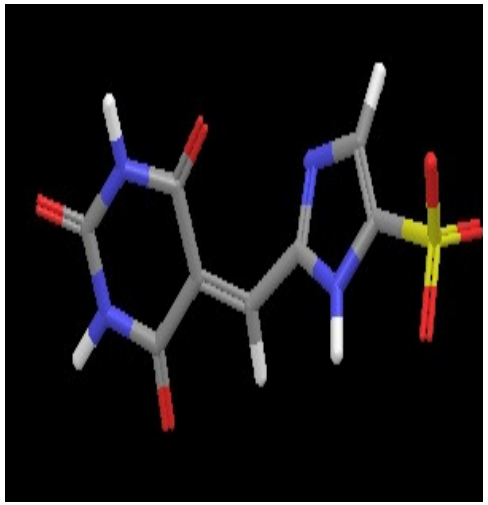


TABLE : 5.1

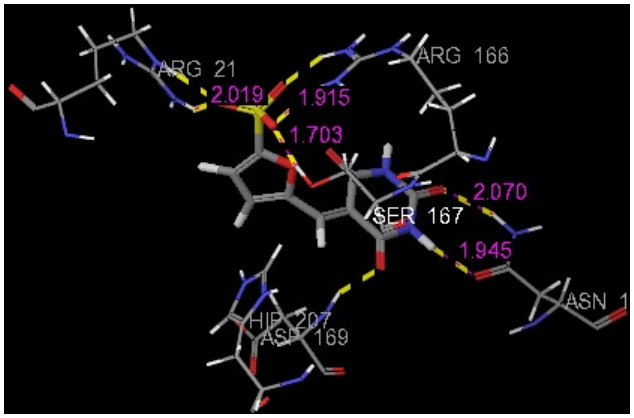
REWARDS

Ligand	G Score	Lipophilic EvdW	PhobEn	PhobEn HB	PhobEn Pair HB	H Bond	Elect	Site map	Pi Cat	ClBr	LowMW
M1	9.23	-1.14	0	0	0	-4.44	-2.97	0	0	0	-0.5
M2	9.22	-1.11	0	0	0	-4.78	-2.84	-1.15	0	0	-0.499
M3	9.15	-1.55	0	0	0	-4.39	-2.85	0	0	0	-0.5
M4	8.28	-1.50	0	0	0	-3.32	-2.19	-0.03	0	0	-0.5

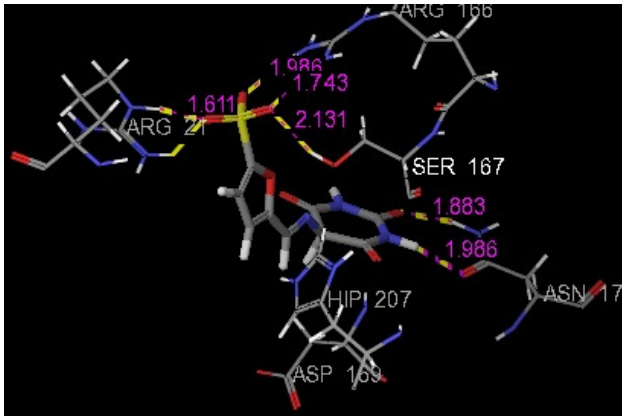
TABLE 5.2 **PENALTIES**

Ligand	Penalties	HB Penal	Phobic Penal	RotPenal
M1	0	0	0	0.095568
M2	0	0	0	0.174767
M3	0	0	0	0.095569
M4	0	0	0	0.095568

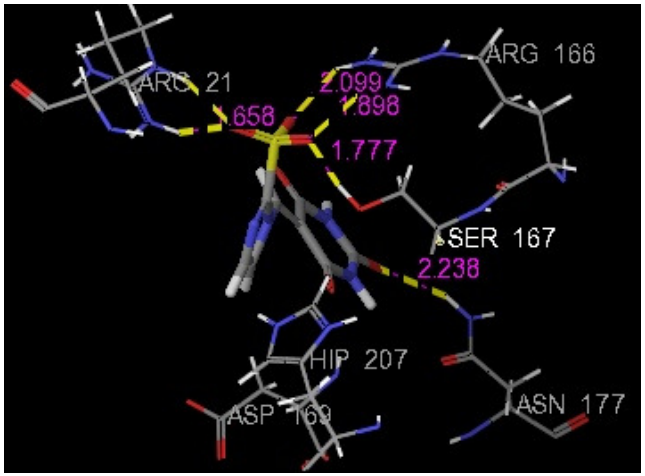
M1


ARG 21
2.019
1.915
ARG 166
1.703
2.070
SER 167
1.945
HIP 207
ASP 169

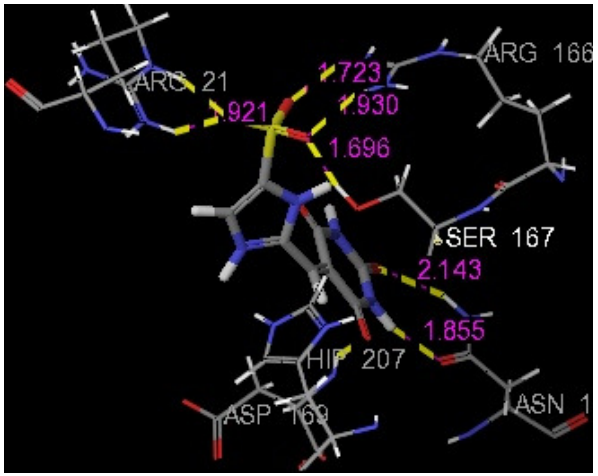
M2


1.611
1.743
ARG 21
2.131
SER 167
1.883
1.986
HIP 207
ASP 169

M3


ARG 21
2.099
1.898
ARG 166
1.658
1.777
SER 167
2.238
HIP 207
ASP 169
ASN 177

M4


ARG 21
1.723
1.930
ARG 166
1.921
1.696
SER 167
2.143
1.855
HIP 207
ASP 169

5.2 SYNTHESIS AND CHARACTERIZATION

Synthesis

The compound M_1 – M_4 were prepared from barbituric acid by various steps using microwave methods. In M_1 and M_4, first step barbituric acid condensed with furfural and 2-Imidazole aldehyde (synthesized by) and the condensed product formed is sulphonated using sulphuric acid. M_2 is prepared by condensing Uramil (5-Amino barbituric acid) with furfural and sulphonating with sulphuric acid. M_3 is synthesized by mannich condensation of barbituric acid, Imidazole and chloroform and the product is sulphonated with sulphuric acid.

Characterization

The synthesized compounds were recrystallized and identified by TLC. The melting point of the products were found and are presented uncorrected. The synthesized compounds were characterized by using sophisticated instruments like IR, NMR, and MASS spectroscopy.

IR SPECTROSCOPY

IR spectra have been used functional groups present in the synthesized compounds.

The presence of impurities like the starting materials i.e. aldehyde, amines were ruled out by ensuring absence of the functional group of the parent compounds.

The synthesized compounds showing following specific characteristic stretching and bending vibrations.

TABLE 5.3

Compound	IR Absorption region (cm^{-1})
M1	3100-3000cm^{-1} (C-H), 1675cm^{-1} (C=C), 1270-1020cm^{-1} (C-C), 1725-1660cm^{-1}(C=O), 910-895cm^{-1}(S-O), 800-600cm^{-1}(C-S), 3500-3300cm^{-1}(N-H), 1070cm^{-1}(C-N).
M2	1725-1660cm^{-1} (C-O), 3500-3300cm^{-1}(N-H), 1070cm^{-1}(C-N), 1600-1430cm^{-1}(C-C), 1300-735cm^{-1}(C=N), 3100-3000cm^{-1}(C-H), 1270-1020cm^{-1}(C-O), 2900cm^{-1}(O-H), 910-895cm^{-1}(S-O).
M3	1725-1600cm^{-1} (C=O), 3500-3300cm^{-1}(N-H), 1070cm^{-1}(C-N), 1600-1430cm^{-1}(C-C)&(C=N), 1300-735cm^{-1}(C-C), 3100-3000cm^{-1}(C-H), 2900cm^{-1}(O-H), 800-600cm^{-1}(S-O).
M4	1725-1600cm^{-1} (C=O), 3500-3300cm^{-1}(N-H), 1070cm^{-1}(C-N), 1600-1430cm^{-1}(C-C)&(C=N), 1300-735cm^{-1}(C-C), 3100-3000cm^{-1}(C-H), 2900cm^{-1}(O-H), 800-600cm^{-1}(S-O).

The two important characteristic have been taken as tangible proof for the formation of the product.

- ✓ 3500-3300cm^{-1} single peak of the (N-H) stretching.
- ✓ 800-600cm^{-1} single peak corresponds to (S-O) stretching.

^{1}H NMR SPECTROSCOPY

The ^{1}H NMR Spectral data of all the synthesized compounds were in conformity with the structure assigned. **TABLE 5.4**

COMPOUNDS	**^{1}H NMR Data** (500 MHz, DMSO-d_6)
M1	δ 2.0 (s, 1H, -OH), 6.2 (s, 1H, Hetero aromatic C-H), 7.4 (s, 1H,Hetero aromatic C-H), 8.01 (s, 1H, -CH), 10.0 (s, 2H, N-H),
M2	δ 2.0 (s, 1H, -OH), 3.3 (s, 1H, C-H), 7.3 (s, 1H, Aldimine-CH), 6.2 (s, 2H, ,Hetero aromatic CH), 10.0 (s, 2H, N-H),
M3	δ 2.5 (s, 1H, -OH), 6.6 (s, 1H, C-H), 7.1 (s, 2H, ,Hetero aromatic C-H), 10.5 (s, 2H, N-H),
M4	δ 2.0 (s, 1H, -OH), 6.6 (s, 1H, C-H), 7.2 (s, 1H, ,Hetero aromatic -CH), 7.7 (s, 1H, ,Hetero aromatic -CH), 10.3 (s, 2H, N-H),

These properties conforms the formation of the products.

^{13}C NMR SPECTROSCOPY

The ^{13}C NMR Spectra further conforms the structure by giving the carbon skeleton present in the synthesized compounds. **TABLE 5.5**

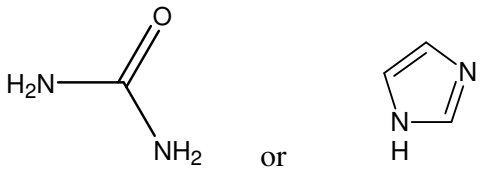
Compounds	**^{13}C NMR Data** (500 MHz, DMSO-d_6)
N_1	δ 39.493,39.660, 39.828, 39.995, 40.162, 40.325, 40.495, 67.102, 84.659.
N_2	δ 39.623, 39.789, 39.956, 40.123, 40.290, 113.235, 150.257, 159.709.
N_3	δ 141.249, 141.332, 152.101, 154.026, 156.149, 158.201.
N_4	δ 77.795, 150.249, 151.332, 151.101,164.026, 166.149, 168.201.

MASS SPECTROMETRY

All the synthesized compounds are futher structurally conformed by molecular ion (M^+) of the varying intensities ascertain the molecular weight of the compounds. **TABLE 5.6**

Compounds	**Mol. for/ Mol wt (calculated)**	**m/e value (Relative abundance)**
M_1	$C_9H_6N_2O_7S$ Mol.Wt.286.22	285.99(**M^+**) (9%), 62.16(**B**) (100%),
M_2	$C_9H_7N_3O_7S$ Mol.Wt.301.23	301.28(**M^+**) (7%), 61.78(**B**) (100%)
M_3	$C_8H_6N_4O_6S$ Mol.Wt.286.26	286.21(**M^+**) (4%), 65.46(**B**) (100%)
M_4	$C_8H_6N_4O_6S$ Mol.Wt.286.26	386.21(**M^+**) (3%), 63.34(**B**) (100%)

The base peaks of the fragments are probably due to



All the above spectral data confirmed the assigned structure of the synthesized compounds.

5.3 BIOLOGICAL SCREENING

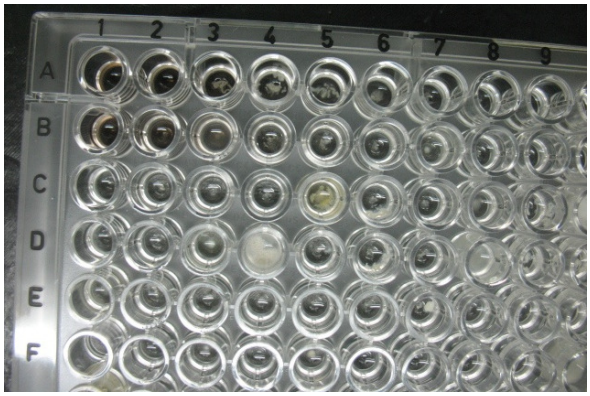
Invitro antitubercular activity

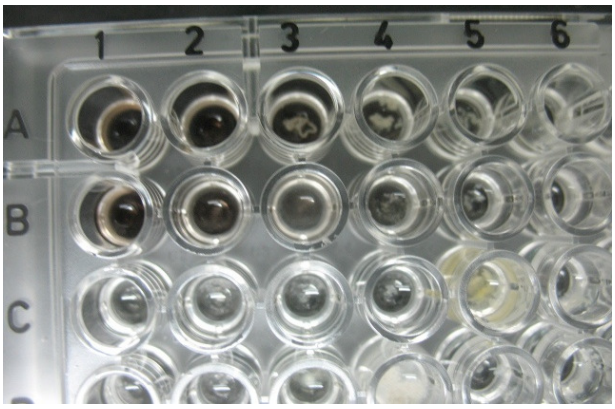
The antimycobacterial activities of the synthesized compounds were determined by the Broth Microdilution method. The organism used for this study is *Mycobacterium tuberculosis* H37RV.

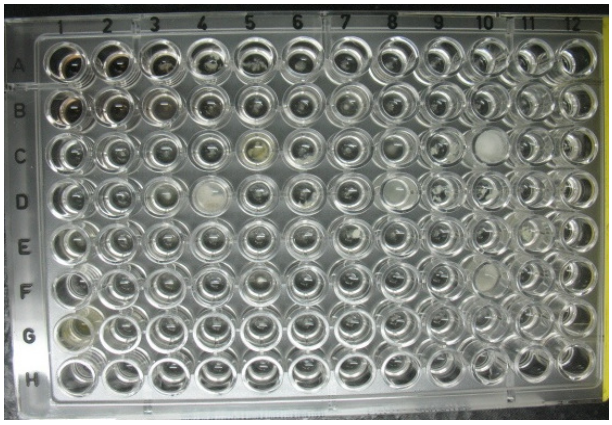
All the synthesized compounds showed antibacterial activity to varying degree against the organism tested. The organism tested was susceptible to compound M1 and M2 and their minimum inhibitory concentration (MIC) was 250µg/ml and 500µg/ml respectively**.** The data pertaining to the activity are presented in the table and the growth of organism was shown in the figure.

Among the synthesized compounds M1 exhibit good activity by minimum inhibition at 250µg/ml and compound M2 at 500µg/ml. M3 and M4 not shown their Minimum inhibitory concentration till 1000µg/ml**. the** result was perfectly correlated with the results. In the docking top scored compound M1 shows best antitubercular activity at lowest concentration 250µg/ml than other compounds. Activity scoring followed by the compound M2 at 500µg/ml and M3 and M4 doesn't show any activity.

Estimation of MIC by Micro broth dilution technique in MiddleBrook 7H9 broth







A – Molecule 1 B – Molecule 1 A

C – Molecule 2 D- Molecule 3

E- Molecule 4 F- INH

G- MTB Control H – Broth control

A row M_1 There is no growth in First and Second well: Concentration till 50 microgram per 200 microliter it was inhibiting the bacterial growth.

C row M_2. It showed activity only in First well; at a concentration of 100 micro liters alone it was effective.

RESULTS **TABLE 5.7**

In vitro antimycobacterial assay of molecules Observation after 21days of incubation at 37ºC for Standard strain H37Rv

	100	50	25	12.5	6.25	3.125	1.562	0.7812	0.3906	0.1953	0.09765	0.4883
Molecule 1	-	-	+	+	+	+	+	+	+	+	+	+
Molecule 2	-	+	+	+	+	+	+	+	+	+	+	+
Molecule 3	+	+	+	+	+	+	+	+	+	+	+	+
Molecule 4	+	+	+	+	+	+	+	+	+	+	+	+
INH	-	-	-	-	-	-	-	-	-	-	-	-
MTB control	+	+	+	+	+	+	+	+	+	+	+	+
Broth control	-	-	-	-	-	-	-	-	-	-	-	-

(-) – absence of Mycobacterial growth (+) – presence of Mycobacterial growth

Recovery Plates: Standard strain H37RV

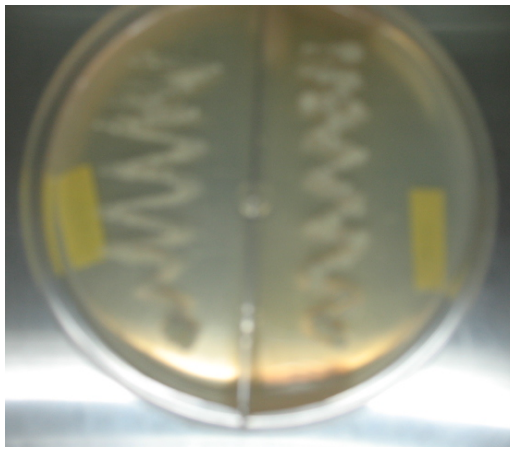
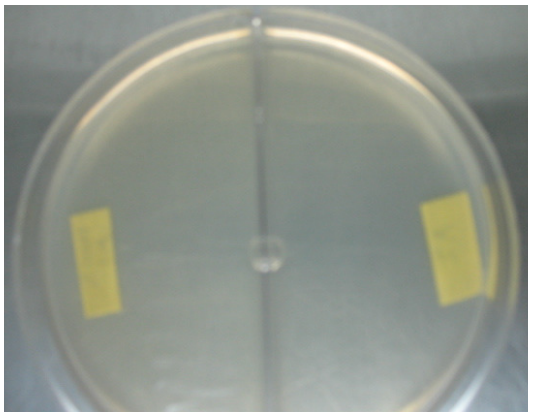


TABLE 5.8

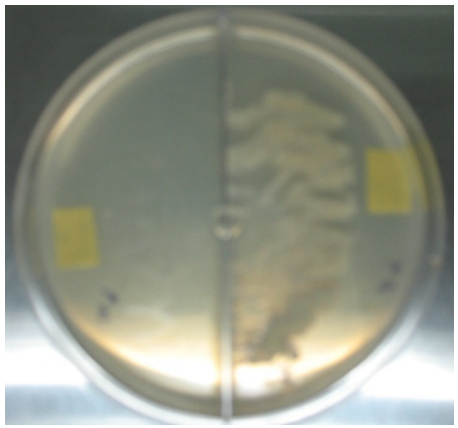
	100	50	25	12.5	6.25	3.125	1.562	0.7812	0.3906	0.1953	0.09765	0.4883
Molecule 1	-	-	+	+	+	+	+	+	+	+	+	+
Molecule 2	-	+	+	+	+	+	+	+	+	+	+	+
Molecule 3	+	+	+	+	+	+	+	+	+	+	+	+
Molecule 4	+	+	+	+	+	+	+	+	+	+	+	+
INH	-	-	-	-	-	-	-	-	-	-	-	-
MTB control	+	+	+	+	+	+	+	+	+	+	+	+
Broth control	-	-	-	-	-	-	-	-	-	-	-	-

(-) – Absence of Mycobacterial growth (+) – Presence of Mycobacterial growth

Molecule M^1 100µg/200µl 50µg/200µl



Molecule M^2 100µg/200µl 50µg/200µl



5.4 ACUTE TOXICITY STUDIES

To assess the safety and toxicity of the synthesized compounds, oral acute toxicity were carried out in female albino mice as per organization for Economic cooperation and Development guidelines 423 (OECD)

The results of acute toxicity studies shown the synthesized compounds are non toxic and did not induce any mortality in the treated mice. The results presented in table.

Evaluation of Autonomic Nervous System

It includes changing in salivation, lacrimation, perspiration, piloerection, micturition and defection and these were found to be normal.

Evaluation of central Nervous system

The animals were observed for ptosis, drowsiness stereotypy, aggression, tremors, convulsion, straup's test, writhing and these were found to be absent. Gait, righting reflex and corneal reflex were found to be normal.

Other interpretations

Skin, fur, eyes, and bodyweight analysis were found to be normal. Tremors, lethargy, diarrhea, and coma were not observed throughout the study period of 14 days.

TABLE5.9

Behavioral and physical observations of synthesized compounds were treated mice for the limit test at 2000mg/kg body weight.

Observation	30min	4hrs	24hrs	48hrs	1 week	2 week
Straub's test	-	-	-	-	-	-
Sedation	-	-	-	-	-	-
Excitation	-	-	-	-	-	-
Jumping	-	-	-	-	-	-
Writhing	-	-	-	-	-	-
Piloerection	-	-	-	-	-	-
Stereotypy	-	-	-	-	-	-
Scratching	-	-	-	-	-	-
Grooming	-	-	-	-	-	-
Aggression	-	-	-	-	-	-
Ptosis	-	-	-	-	-	-
Exopthalmia	-	-	-	-	-	-
Loss of righting reflux	-	-	-	-	-	-
Loss of pinnal reflux	-	-	-	-	-	-
Loss of corneal reflux	-	-	-	-	-	-
Salivation	Normal	Normal	Normal	Normal	Normal	Normal
Lacrimation	-	-	-	-	-	-
Skin and fur	Normal	Normal	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal	Normal	Normal
Tremors	-	-	-	-	-	-
Diarrhea	-	-	-	-	-	-
Coma	-	-	-	-	-	-

SUMMARY

&

CONCLUSION

SUMMARY AND CONCLUSION

- Candidate molecules were designed based on HipHop (catalyst® software) and docked against Thymidylate Synthase X (ThyX) protein using Drug Design Software (Maestro® 9.1).

- The SAR of high scoring molecules was studied and they are optimized based on the synthetic feasibility.

- Four compounds with high G score were shortlisted for the synthesis and they are labeled as M_1, M2, M3, and M4.

- The structures of the compounds were assigned on the basis of IR, ^{1}H NMR, ^{13}C NMR, and MASS data.

- ***In-vitro*** **Anti tubercular activity –** The compounds were screened for antitubercular activity by Broth Micro dilution method.

- Of the four compounds M1 and M2 exhibited antitubercular activity; compound M1 exhibited minimum inhibition at 250µg/ml and M2 at 500µg/ml.

- The docking score of the candidates correlates with the *invitro* activity of the ligands.

- **Toxicological evaluation -** The results of acute toxicity studies have shown that the synthesized compounds are non toxic and did not induce any mortality at the highest OECD guideline dose 2000mg/kg.

These compounds are attractive strong point to find newer lead compounds which will find ultimate use in the treatment of drug resistant tuberculosis. Further these compounds are tested for advanced antitubercular assays in *in-vitro* like Luciferase Reporter Phage Assay (LRP), Micro plate Alamar Blue Assay (MABA), Resazurin microtitre Assay (REMA),

Serine/Threonine Protein (STP) Kinase Assay, BACTEC-TB system and *In-vivo* assay like Balb/C mouse model for CFU and mortality.

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